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PATHOPHYSIOLOGY OF ANTICHOLINESTERASE AGENTS

Annual Report

John E. Rash, Ph.D.
Julie K. Elmund, Ph.D.

May 9, 1986

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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Colorado State University
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John E. Rash, Ph.D., Julie K. Elmund, Ph.D.

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In this second Annual Report, we describe the acute, delayed, and long-term effects on rat neuromuscular junction (NMJ) ultrastructure and physiology following single acute injections of very low to near lethal doses of physostigmine, a reversible anticholinesterase (anti-ChE) compound. In addition, we describe the immediate and long term effects of subacute exposures at doses which produce sustained blood ChE inhibitions of 40% (±10%) and 80% (±10%) for up to 14 days and of the reversibility of effects during recovery for 3-28 days following termination of subacute exposure. In this in-depth study, we describe dose-response curves for enzyme inhibition, ultrastructural pathology, and altered physiology, as well as data pertaining to reversibility of toxic effects following both acute and subacute

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exposures. In parallel physiological experiments, effects of subacute physostigmine administration appeared minimal and quickly returned to normal during the recovery period after Alzet pump removal, supporting the data obtained by ultrastructural analysis. Finally, we present preliminary data showing that the threshold for producing neuromuscular pathology is lowered by stress and/or by sustained neuromuscular activity. These data demonstrate that plasticity, repair, and recovery of function of nerve terminals, muscle plasma membranes, and muscle cytoplasm occur very rapidly in the rat. However, the occurrence of delayed destructive changes in a few myofibers suggests the need for continued cautious evaluation of the secondary repair and remodeling processs.

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I. SUMMARY

In this Second Annual Report, we continue our descriptions of the acute, delayed, and long-term effects on mammalian neuromuscular junction (NMJ) ultrastructure and physiology following very low to near lethal dose single injections of the anticholinesterase compound, physostigmine. In addition, we describe the effects of subacute exposures at doses which produced sustained serum ChE inhibitions of 30-50% and 80% for up to 14 days. In this in-depth study, we also describe dose response curves for enzyme inhibition, ultrastructural pathology, and altered physiology, as well as data pertaining to reversibility of toxic effects following both acute and subacute exposures. Finally, we present data showing that the threshold for producing neuromuscular pathology is lowered by sustained neuromuscular activity.

In the first year, the LD_{50} of physostigmine was determined by bioassay to be approximately 0.75 mg/kg for adult male albino rats. Three treated and two control rats were then administered single subcutaneous injections of physostigmine in dilute acetic acid "carrier" solution (dose levels of 0.001 to 1.1 LD_{50}) or a "sham" injection using "carrier" solution (control). Immediately before injection and at ½ hour, 1, 7, 14, 28, and 56 days post injection (PI), samples of diluted whole blood were obtained for determination of cholinesterase enzyme inhibition (over 2,900 enzyme assays). Rats were fixed by whole body perfusion at the desired intervals PI, and the diaphragm, soleus, and extensor digitorum longus (EDL) muscles removed for examination by conventional transmission electron microscopy. Changes in muscle and nerve ultrastructure and physiology were then correlated directly with changes in blood cholinesterase inhibition levels for the same rat. At ½ hour PI, blood ChE enzyme inhibitions were measured at approximately 25% at 0.001 LD_{50}, 33% at 0.01 LD_{50}, 67% at 0.1 LD_{50}, 75% at 0.3 LD_{50} and 89% at 0.8 LD_{50}. At low and very low doses (0.001 and 0.01 LD_{50}), we observed no evidence for neuromuscular pathology in any muscle at any time from ½ hr to 56 days PI. Thus, low levels of physostigmine appeared to produce no major ultrastructural alterations at the NMJ. At 0.1 LD_{50} (67% ± 10% inhibition), a few fibers in the diaphragm (but none in EDL or Sol) showed the first limited signs of neuromuscular toxicity, (i.e., slight swelling of post-junctional mitochondria, nuclear membranes, and golgi cisternae). However, ½ hour after single high dose injections of 0.8 LD_{50}, all neuromuscular junctions of the constantly-used diaphragm and soleus myofibers exhibited supercontraction of sarcomeres in the subjunctional sarcoplasm. Often, Z bands were missing, free thick and thin filaments were present in disorganized masses, and a mixed population of "frothy" and grossly distended mitochondria were observed disrupting the subjunctional sarcoplasm. EDL muscles from the same rats were much less affected at ½ hr but exhibited greatly increased damage by 24 hrs. We conclude that the delayed damage to EDL fibers is related to the resumption of voluntary myofiber activity during the initial 6 to 12 hour period of low cholinesterase activity. In addition, the "threshold" for producing severe endplate

pathology was more precisely defined by acute injections of additional rats with 0.3 LD_{50} (75% serum ChE inhibition). Sixty percent of diaphragm myofibers but 0% of unstimulated soleus and EDL myofibers were supercontracted.

In our analysis of "dose/response" relationships for physostigmine over the range 0.001 to 0.1 LD_{50}, we have obtained additional evidence for a relatively narrow "threshold" for the induction of severe cytopathology at the neuromuscular junction. We show that the threshold for supercontraction/endplate damage may be altered by stress and/or increased neuromuscular activity. We propose a more detailed sequence of subcellular changes during acute physostigmine toxicity:

Near threshold (i.e., at about 70% inhibition with moderate neuromuscular activity):

1) Appearance of distended or swollen subjunctional cytomembrane systems (Golgi cisternae and vesicles, nuclear membranes, and endoplsmic reticulum), but no distention of sarcoplasmic reticulum,

Threshold (i.e., at about 75% inhibition and with moderate neuromuscular activity; four distinct stages detectable)

2) Distention of sarcoplasmic reticulum in the immediate subjunctional sarcomeres,

3) Slight swelling of nerve terminal and subjunctional mitochondria,

4) Appearance of "blisters" in subjunctional mitochondria,

5) Subjunctional mitochondria become "frothy" (i.e., develop numerous internal blisters),

Above threshold (i.e., at about 80% inhibition and with moderate neuromuscular activity; four distinct stages discernable):

6) Gross distention of subjunctional mitochondria; swelling of nerve terminal mitochondria, indicating increased nerve terminal activity due to substantial failure of other motor units.

7) Supercontraction of subjunctional sarcomeres.

8) "Explosion" of subjunctional mitochondria (some may exhibit obvious membrane discontinuities), and finally,

9) Disappearance of Z bands and dissolution of myofilaments.

During recovery from doses of 0.8-0.1 LD_{50}, the destructive effects observed in all myofibers of diaphragm and soleus were partially reversed 24 hours PI, and blood ChE levels had returned to near normal. By seven and 14 days after injection of 0.8-1.1 LD_{50}, most myofibers had regained normal ultrastructure. However, in diaphragm and soleus myofibers, localized regions of junctional folds within individual endplates were devoid of attached nerve terminal branches while other fibers showed evidence for collateral sprouting and renewed synaptic remodeling and/or synatogenesis. These relatively infrequent endplate alterations suggest to us that endplate repair and/or habituation processes are extremely rapid in most fibers, but that the more severely affected fibers may compensate for severe endplate damage by (partial) denervation and reinnervation via the observed small collateral "sprouts."

In **subacute exposure experiments**, we have found that low doses (yielding sustained 30-50% inhibition) produce no detectable alterations in endplate ultrastructure at 3, 7 or 14 days.

Likewise, at relatively high dose subacute exposures (80% sustained serum ChE inhibition), most fibers exhibited no obvious changes in endplate fine structure. However, after 7 days, a few fibers in the soleus muscle had evidence for sarcomere damage and partial repair. No such alterations were observed in Diaphragm or EDL. At 14 days, many fibers appeared "edematous", that is, their nuclei were swollen and had only a very thin band of peripheral heterochromatin, and the subjunctional cytoplasm was 50% to 100% thicker than in control. However, sarcomeres and other cytomembranes appeared normal. In parallel physiological experiments, effects of subacute physostigmine administration appeared minimal. For both high (80% inhibition) and low (30% inhibition) dose Alzet pump exposure, EDL average twitch tensions and ability to sustain 20 hz contraction were within normal range. A slight tendency toward increased fatigability with prolonged stimulation was observed during subacute administration, but this was quickly reversed during the recovery period after Alzet pump removal.

Finally, we show that muscle use may be an important factor in drug toxicity. Data obtained from stressed and physiologically stimulated animals suggests that the threshold for expression of physostigmine toxicity may be lowered substantially by concurrent muscle activity. This may help to explain why the constantly used diaphragm myofibers (whether fast twitch, slow twitch, or intermediate) are most rapidly and severely affected in unstimulated/unstressed animals exposed to near lethal doses of physostigmine.

The process of prolonged endplate depolarization associated with normal neuromuscular activity appears to cause the observed rapid destructive supercontraction ½hr PI. During recovery, most fibers quickly return to near normal and show no obvious residual damage after 7-14 days. However, partial denervation and reinnervation by collateral sprouting occurs in a few myofibers. Thus, these data demonstrate that plasticity, repair, and recovery of function of nerve terminals, muscle plasma membranes, and muscle cytoplasm occur very rapidly in the rat. However, the occurrence of delayed destructive changes in a few myofibers suggests the need for continued cautious evaluation of the secondary repair and remodeling processs.

II. FOREWORD

"Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations."

In conducting the research described in this report, the investigator(s) adhered to the "Guide for Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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VI. BODY OF REPORT

A. INTRODUCTION

STATEMENT OF PROBLEM

During the three years of this contract, we are to use ultrastructural, physiological, and biochemical techniques to complete four major tasks, each having several components:

Task 1, Year 1 (Supercontraction/Fiber Type Specifity)

a) Establish whether or not acute administration of physostigmine produces supercontraction of muscle fibers in the rat and, if so,

b) establish whether or not the supercontraction or other damage is related to muscle fiber type (fast or slow twitch muscles).

Task 2, Year 1 (Habituation)

a) Establish whether subacute administration of physostigmine produces physiological, biochemical, and/or ultrastructural changes consistent with **habituation** or

b) establish if the subacute effects are similar to acute or to delayed effects.

Task 3, Years 1, 2, and 3 (Dose Response Relationships)

Establish the dose response relationships for

a) acute physostigmine exposure,

b) Ascertain if supercontraction also occurs in guinea pig myofibers at near LD_{50} dose,

c) delayed changes resulting from a single exposure to physostigmine, and

d) chronic physostigmine exposure.

Task 4, Years 2 and 3 (Reversibility)

a) Establish whether or not acute effects of physostigmine (physiological and/or ultrastructural) are reversible.

b) if so, establish the time course of such reversibility;

c) to establish whether or not chronic effects of physostigmine (physiological and/or ultrastructural) are reversible and

d) if so, to establish the time course of the reversibility.

B. OVERALL SCOPE OF THE WORK

The techniques to be used include, where appropriate, morphometric light microscopy and thin section transmission electron microscopy. We are to pay particular attention to pre- and post-synaptic alterations of neuromuscular junctions, especially evidence for supercontraction; damage to membrane-bound organelles such as mitochondria, sarcoplasmic reticulum, and T-tubules; changes in nerve terminal size, number of synaptic vesicles; and/or alterations in nerve terminal mitochondrial morphology. We are to determine if the diaphragm, soleus, or extensor digitorum longus (EDL) muscle is the best model system for assessing ultrastructural alterations. Blood and/or tissue cholinesterase levels are to be measured periodically in each animal as an independent measure of the effect and distribution of physostigmine. Physiological studies are to include an assessment of twitch potentiation of EDL muscles following acute and subacute exposure to physostigmine and, where possible, are to include an initial assessment of the afferent and efferent components of the spinal reflex arc following acute exposure to physostigmine.

C. BACKGROUND/REVIEW OF LITERATURE

The prototype anticholinesterase agent, physostigmine (or eserine), is a toxic tertiary carbamate alkaloid isolated from the "Calabar bean" or "esere nut" of Physostigma venosum (1). Systematic investigations of its chemical structure/activity relationships led to the introduction of Neostigmine, which is now used in the symptomatic treatment of myasthenia gravis (1). The current major clinical application of physostigmine is in the treatment of glaucoma (1) and in the symptomatic treatment of atropine intoxication (1).

1. PRIMARY TOXICITY OF ANTICHOLINESTERASE AGENTS ATTRIBUTED TO DEPOLARIZING NEUROMUSCULAR BLOCKADE

It has been known for at least 60 and 75 years, respectively, that the anticholinesterase agents, neostigmine and physostigmine, and the depolarizing neuromuscular blocking agents, nicotine and succinylcholine, produce profound myopathies and neuropathies, as well as distinctive pathophysiologies of neuromuscular transmission (2-23). Both are now known to be due primarily to severe and prolonged endplate depolarization. Koelle has suggested that "to exert a significant effect in vivo, an anti-ChE agent must generally inhibit from 50 to 90% of the functional AChE at a given site" (1). In support of this assertion, Hudson (24) showed that with pyridostigmine, severe morphological alterations (supercontraction of subjunctional sarcomeres) do indeed occur at blood cholinesterase inhibitions above approximately 70%. However, she also reported that subtle alterations of nerve terminal components (especially mitochondria) occur at 0.001 LD_{50} or at a serum enzyme inhibition of less than 10%. Unfortunately, these low dose alterations (swollen mitochondria in the nerve terminal) are especially difficult to interpret because anticholinesterase agents are also known to produce profound bradycardia, hypotension, bronchoconstriction, and release of epinephrine, resulting in substantial systemic tissue hypoxia (1). The resulting increased metabolic rate and lowered O_2 tension in the nerves and muscles may also produce severe mitochondrial swelling, especially when fixed by glutaraldehyde (25-27 and 57). Consequently, the precise fixation techniques used by Hudson and coworkers may not have allowed unambiguous identification of the sources of the proposed changes. Thus, it is not yet established whether the reported changes at low doses represent subtle primary and/or secondary effects of anticholinesterase agents or artifacts of glutaraldehyde fixation.

2. ULTRASTRUCTURAL STUDIES OF ANTICHOLINESTERASE TOXICITY

Numerous ultrastructural studies of acute anticholinesterase toxicity reveal profound alterations of the neuromuscular junction at near LD_{50} doses (7-23). These include supercontraction of subjunctional sarcomeres with rapid progression to Z band disruption and myofibril disassembly, swelling and explosion of mitochondria in the nearby subjunctional sarcoplasm, and in the nerve terminals, relatively severe mitochondrial swelling. After a single high dose exposure, these initial changes appear to be ameliorated within a few days.

However, additional delayed effects are noted, including 1) at about 14-56 days, destruction and removal of some junctional folds and their replacement by vesicular debris similar to that seen in the autoimmune disease, myasthenia gravis (**11,28-31**); 2) at 7-28 days, the disappearance of some nerve terminal branches, yielding areas of junctional folds devoid of associated nerves as is found in other types of partial endplate denervation (**32**), and 3) at 14-56 days, the formation of small diameter nerve terminal branches ("collateral sprouts") similar to those seen in myasthenia gravis (**11,32,33**) and in human neurogenic neuromuscular diseases (**34**).

In our previous studies of Neostigmine toxicity (**20,21**), we also described conventional transmission electron microscopy and intracellular recording techniques to show that these anti-ChE-treated endplates had severe pre- and post-synaptic alterations (as described above) and that these alterations persisted with "chronic" (multiple injection) exposures. No long-term recovery or acute-delayed effects were described in those reports. This study addresses several of those deficiencies (ie: "acute-long term recovery", subacute (or "chronic") exposure, and "subacute-recovery" as well as providing enzyme histochemistries and dose response relationships for each of the exposure regimens.

3. Summary of First Annual Report

In the first year, we 1) established the LD_{50} of physostigmine as 0.8 mg/kg; 2) injected twenty four groups of 3 "experimental" and 2 "control" rats with 0.001 LD_{50} to 1.1 LD_{50} and obtained samples for electron microscopic analysis from diaphragm, soleus and EDL muscles ½hr and 1, 7, 14, 21, and 56 days PI; 3) established regimens for obtaining continuous levels of blood ChE enzyme inhibitions of 30-50% ("low dose") and 70-90% ("high dose") using Alzet mini-osmotic pumps; 4) obtained diaphragm, soleus, and EDL muscles from these "subacute exposure" groups for ultrastructural analysis of possible changes at 3, 7, and 14 days post implantation and during recovery at 3, 7, 14 and 28 days after surgical removal of the pumps; 5) Obtained blood samples for ChE inhibition analysis before drug exposure and at ½hr and 1, 3, 7, 14, 28 and 56 days PI until the animals were fixed for electron microscopy; 6) identified a synergistic effect of anesthesia and physostigmine on muscle physiology; 7) performed initial measurements of the effect of low to high doses (0.001 to 0.8 LD_{50}) of physostigmine on muscle twitch tension and resistance to fatigue; 8) performed ultrastructural analyses of acute high dose exposures; 9) obtained preliminary ultrastructural data from low to moderate dose exposure, and 10) obtained preliminary data concerning "reversibility" of cytopathological alterations from 1 day to 56 days following a single acute injection. Because most of these projects continued into Year II, (and hence, were not complete as described in the first Annual Report), those incomplete data and conclusions are not separately summarized in this section. Rather, brief summaries of previous data (as well as a few electron micrographs previously provided) are presented in each of the appropriate sections. Note, also, that much of the "Rationale" and "Methods" sections are unchanged from the previous report.

D. RATIONALE

Following exposure to the "irreversible" anti-ChE agent, Soman, endplate AChE is "permanently" inactivated (potentially until new AChE is synthesized). With normal respiratory activity, virtually continuous endplate depolarizations ensue, thereby causing the death of the test animal by respiratory failure.

The quaternary carbamate anticholinesterase, pyridostigmine, has been shown to be a moderately effective prophylactic agent against exposure to lethal doses of Soman (35). The protective mechanism(s) is (are) entirely unknown. However, it has been suggested that the "reversible" anticholinesterase agents competitively occupy the esterase sites during exposure to the "irreversible" anticholinesterase agents. After termination of exposure to Soman plus a "reversible" anti-ChE agent, the "protected" AChE site is suggested to be "unmasked" by hydrolysis of the reversible agent, leading to sufficient reactivation of acetylcholinesterase (AChE) to permit recovery of muscle function. However, this proposal leads to a dilemma: In the presence of <u>two</u> powerful anti-ChE agents, it is not clear how the more vital muscle activities (breathing, for example) are to continue during the prolonged period required to reactivate AChE. This reactivation period is estimated to be at least 30 minutes for Neostigmine and substantially longer for physostigmine and pyridostigmine (1). Clearly, if the <u>only</u> mechanism of action were based on competition for and co-blockade of a single class of AChE molecules, death would ensue from the prolonged neuromuscular blockage produced by either/both agents. Since a substantial number of rats survive a dose of several LD_{50}s, the protective mechanism(s) must be other than that which has been proposed.

In an attempt to clarify the protective mechanisms afforded by "reversible" anti-ChE agents, this "in-depth" study will utilize biochemical, ultrastructural, and physiological approaches to identify the acute and delayed effects of very low to near lethal doses of physostigmine. In these experiments, the rat is used as a model system for analyzing chemoprophylactic agents for nerve agent exposure because, of all animals tested, the rat is among the <u>least</u> protected by prior or concurrent exposure to the "reversible" anti-ChE agents (35,36). Using the rat as our primary model system, we are investigating the immediate and long-term effects of single acute injections, determining the dose-response curve for those alterations, and measuring the extent of reversibility of those toxic effects. In addition, we are examining the effects of <u>subacute</u> exposure on endplate ultrastructure, serum cholinesterase activity, and muscle physiology over a two-week subacute exposure period and during recovery, for up to 28 days after termination of the subacute exposure. These data are expected to provide new insights into the mechanism of action of this potent anti-ChE agent, as well as the extent of the reversibility of its toxic effects. From these data, it may be possible to make a more informed decision concerning its possible use as a prophylactic agent against the irreversible nerve agent.

E. EXPERIMENTAL METHODS

1. DEVELOPMENT OF CARRIER SOLUTIONS FOR PHYSOSTIGMINE

Since physostigmine is very labile above pH 6 (37), for subacute exposure experiments, physostigmine solutions must be made and maintained under slightly acidic conditions. Moreover, to facilitate comparision of acute and subacute exposure regimens, similar carrier solutions were devised for use in all experiments. Physostigmine solutions and the solutions used in the "sham injection" ("control") experiments contained glacial acetic acid diluted 1:2000 (i.e., 10 mM). These dilute acetic acid solutions were well tolerated by the animals and did not produce evidence of tissue damage or of necrosis at the site of injection or implantation, nor did they produce any detectable alterations of endplate morphology in any of the muscles analyzed.

2. ESTABLISHMENT OF LD_{50} FOR ACUTE EXPOSURES

For rats weighing 180-250 gm, the LD_{50} of physostigmine was determined during Year I to be approximately 0.75 mg/kg (see previous report).

3. PROCEDURE FOR ACUTE EXPOSURE TO PHYSOSTIGMINE

Seventy-eight male Wistar rats, weighing 180 to 250 g were given single subcutaneous injections of 0.001 to 1.1 LD_{50}. Acute exposures were made by single injections in the rostral mid-back region. Blood samples were obtained from experimental and control rats immediately before injection and at 30 minutes, 1, 7, 14, 28, and 56 days PI and coded for subsequent "double blind" analysis and correlation with the tissue samples that were examined ultrastructurally. Two sham injected "control" rats were obtained for examination after each of the same exposure intervals (30 minutes to 56 days PI; age and sex matched controls).

4. SUBACUTE ADMINISTRATION OF PHYSOSTIGMINE

a. Determination of Doses Required to Obtain Pre-Determined Enzyme Inhibition Levels

During Year I, the doses and implantation regimens required to obtain 30% and 80% blood cholinesterase enzyme inhibition were determined. Exposure levels of 0.25 mg/ml and 25 mg/ml delivered at a rate of 2.5 µl/hr yielded sustained serum ChE inhibitions of 30-50% and 70-90%, respectively. These values are equivalent toa delivery of 0.003 LD_{50}/hr and 0.3 LD_{50}/hr. Nevertheless, this 100-fold difference in drug delivery with only a 2-3 fold difference in blood ChE inhibition is consistent with data from acute exposure experients.

As in the previous experiments, blood enzyme levels were measured before implantation of the pumps and after 24 hours, 2, 5, and 14 days exposure and in the subacute-recovery experiments at 7, 14, and 28, days after pump removal.

To confirm the validity of the enzyme inhibition measurements, the pumps were removed from each animal before perfusion fixation and their contents and pumping rates were examined by four independent methods:

a) volumetric and weight measurement of residual pump contents,

b) examination of solution for tell-tale changes in color associated with loss of biological activity,

c) double blind analysis for necrosis or other signs of surgical fault at the implant site, and in cases with questionable results,

d) re-injection of residual pump contents into untreated animals and measurement of the resulting ChE enzyme depressions. Pumps that were found not to have released the proper dose of physostigmine were identified and the corresponding animals discarded from the study. (Note: In preliminary experiments, we found that pump failure occurred only when the implantation cavity was not sufficiently large. The resulting compression of blood vessels in the skin over the pump resulted in inadequate vascular perfusion and localized tissue necrosis, and occasionally, in partial pump blockage or inadequate vascular perfusion for normal drug absorption.) In those cases, replacement animals were obtained and processed according to standard protocols.

5. MUSCLE TYPES EXAMINED BY ELECTRON MICROSCOPY

Muscles composed primarily of slow twitch fibers have been reported to be damaged much more severely by anticholinesterase agents than those composed primarily of fast twitch fibers (12,13,20). Therefore, we chose to compare the effects of physostigmine on muscles with relatively pure populations of **fast twitch** (Extensor digitorium longus or EDL muscle) and of predominantly **slow twitch** (soleus) myofibers. However, it is possible that the relevant factor in expression of anti-ChE toxicity is not twitch contraction time (38-41) or pattern of firing of fast twitch vs. slow twitch fibers (i.e., phasic vs. tonic activation as suggested by Ward (13), but may instead be related to the amount/duration of muscle activity. Thus, as a counter example to muscles with nearly pure fiber types, the **diaphragm** muscle was also selected because it is a **"mixed"** muscle containing fast, intermediate, and slow twitch fibers (38-40). The close proximity of three fiber types in the same muscle provides for an internal fiber-type "control." Presumably under respiratory insufficiency, all fiber types (fast, slow, and intermediate) are activated continuously at near maximum levels. The "mixed" diaphragm muscle may then be contrasted with the fast twitch EDL muscle, in which the fibers are voluntarily rested during the initial period of neuromuscular distress following physostigmine injection. In respiratorily distressed rats, even the fast twitch fibers in the diaphragm have relatively continuous firing, presumably greater even than that seen in the postural muscles such as in the slow twitch soleus (41). Under these conditions both diaphragm and soleus muscles are relatively continuously activated until the animal collapses from exhaustion/muscle failure. However, even then, the diaphragm myofibers must continue to contract lest the animal die of respiratory failure. Thus, these three distinctive types of muscles (EDL, soleus, and diaphragm) provide a wide variety of patterns of muscle activity, thereby facilitating the evaluation of normal muscle use on acute physostigmine toxicity.

6. FIXATION FOR ELECTRON MICROSCOPY

All drug-treated and sham injected "control" rats were fixed for electron microscopic examination by arterial perfusion via the left ventricle. The initial perfusate (30 seconds to 2 minutes) consisted of a 39°C rat Ringer's solution containing 10 units/ml of heparin. All solutions were pressurized with 95% O_2, 5% CO_2 at 150 mm Hg (to minimize O_2 loss as occurs when solutions are pressurized with N_2 gas or by equilibration with normal atmosphere). After tissue "clearing," a solution of oxygenated rat Ringers buffer containing 2.5% glutaraldehyde (pH 7.2) was perfused for approximately 5 minutes. [Note: In preliminary experiments using control animals, we have determined that using 100% nitrogen gas to provide pressurization for the perfusion apparatus (**24**) substantially decreases O_2 tension in the perfusate, resulting in severe mitochondrial swelling as a fixation artifact (**25, 57.**)]

Muscle samples were removed for electron microscopy only when a) glutaraldehyde-induced rigor ensued within 30 seconds, b) the liver and kidneys were uniformly blanched and yellowed within 2 minutes, and c) exposed muscles were uniformly yellow (usually within 1-2 minutes). Whole EDL and soleus muscles and strips of diaphragm were immediately removed and placed in vials containing 2.5% glutaraldehyde in Ringer's buffer. The vials were then placed in an ice bath or in the refrigerator (0-4°C) for 1-24 hours.

The quality of perfusion fixation of each muscle sample was graded from "1" (poor) to "10" (excellent) as it was removed from the rat. We previously reported that small regions of inadequate perfusion (as confirmed by presence of blood cells in capillaries) were occasionally found in the highest rated perfusions, and areas of "adequate" fixation were found even in the "poor" perfusions. Thus, we have established the following ultrastructural criteria for accepting any sample for detailed morphological analysis: Extra junctional and non-muscle mitochondria have no or very few dilations or internal swellings, nuclear and SR membranes devoid of swellings or blisters, and no erythrocytes in the capillaries within 250μm of the NMJ's.

7. LOCATING ENDPLATES; EMBEDDING SAMPLES IN PLASTIC

In the first year report, we described the major artifacts resulting from immersion fixation or from poor perfusion fixation, many of which have been misinterpreted by other groups as drug effect. Such poor perfusions usually are recognized by the presence of numerous erythrocytes (RBCs) in most capillaries and by the presence of characteristic "swollen" mitochondria in all area of myofibers, in the nerve terminals, and in their myelinated axons. Criteria for recognizing artifacts were described and in the 1st Annual report and in Lee,et.al. (57) and will not be repeated here.

After primary glutaraldehyde fixation, endplate regions were stained using a modified in vitro acetylcholinesterase stain (42,43). "Control" endplates were visible within 1-2 hours, whereas endplates from "high dose" anticholinesterase treated animals were stained for 18 to 24 hours. (We attribute this delayed staining to the slow hydrolysis of the physostigmine originally bound to endplate ChE and the subsequent "reactivation" of AChE [1] to yield positive staining.) Small segments containing opalescent endplates were dissected free and post-fixed in OsO_4 for 1 hour. Samples were then stained in 0.5% aqueous unbuffered uranyl acetate for 16 to 20 hours, dehydrated in a graded ethanol series, transferred through a graded acetone-plastic series, and embedded in one of two plastic mixtures: a) 10% Epon 812, 20% Araldite 6005, 70% dodecenyl succinic anhydride with 1.5% DMP-30 as catalyst or b) in Spurr's plastic (44), and polymerized at 70°C for 24 hours. (In Year II, all samples were embedded in Spurr's plastic.)

8. MICROTOMY AND ELECTRON MICROSCOPY

Gray to pale gold sections were cut with EMS or Dupont diamond knives using Sorvall MT-2B or LKB Model IV ultramicrotomes, collected on 200 or 700 mesh copper grids, and post-stained with lead citrate (45,46). Specimens were examined at 80 kV on Philips EM 200 or Philips 400T transmission electron microscopes and photographed at initial magnifications of 1,000X to 40,000X using Kodak Electron Microscope Film. Thick sections were stained with toluidine blue and photographed at 400-900X using a Zeiss Photoscope.

Initially, all samples for electron microscopic analysis were coded and examined "double blind." At least three (to as many as 12) endplates from each muscle obtained from each treatment regimen or "control" were photographed. To date, over 575 endplates have been photographed, yielding more than 1825 electron micrographs (See Table I, p. 17). This procedure was maintained during initial photography and preliminary evaluation, but was discontinued as distinctive features of drug cytotoxicity were identified and catalogued. However, to avoid secondary (discretionary) bias, especially in the evaluation of the low-dose samples, every endplate identified in every sample examined was photographed for detailed ultrastructural analysis.

TABLE I. Sources of Ultrastructural Data

	Acute	Subacute	Muscle Use	Total
# of rats in study	120	70	8	270
# of test groups (5/group)	24	14	NA	38
# of rats examined to date	60	25	5	115
# of blocks sectioned	150	75	18	243
# of ChE Assays	1450	800	4500	2900
# of endplates photographed	500	80	25	605
# of TEM's	1500	225	100	1825

9. WHOLE BLOOD CHOLINESTERASE ENZYME ASSAY

We have used the radioisotope assay method of Siakotos, et al (47) to determine blood cholinesterase enzyme activity/inhibition levels. Using ^{14}C-acetylcholine as the substrate, we have measured the relative activity of blood cholinesterase after acute and subacute exposure to physostigmine. Samples were obtained before injection of physostigmine and at 30 minutes, 1, 7, 14, 28, and 56 days PI. Two 50 µl samples of whole blood were drawn into heparinized capillary tubes from a small incision in the tail vein. Samples were diluted 1:9 (50 µl: 450 µl) with distilled water, mixed thoroughly, then frozen in 1 ml plastic vials by immersion in LN_2. For enzyme assays, individual samples were thawed, and within 3 minutes of thawing, injected into the enzyme reaction mixture.

According to the method of Siakotos et al (47) blood ChE ^{14}C-enzyme activity is determined by the measuring the amount of ^{14}C-acetate generated from ^{14}C-acetylcholine after five minutes in the reaction mixture. Diluted whole blood (100 µl) was added to 100 µl of 0.1 M sodium phosphate (pH 7.38) and 100 µl of ^{14}C-acetylcholine (containing 0.3 µmoles of total acetylcholine and 0.10 µCi of ^{14}C-acetyl choline). This solution was mixed thoroughly and incubated at 25°C. The reaction was stopped by adding excess resin-dioxane mixture to reach a volume of 5 ml. The resin-dioxane slurry was prepared by adding 200 ml of dioxane to 50 g of AG 50W-X8 cation exchange resin (200-400 mesh from Biorad). The resin was previously converted to the Na+ form, washed with acetone, and dried <u>in vacuo</u>. Five ml of dioxane was added to this mixture, mixed thoroughly, and then centrifuged at 1500xg max for two minutes. One ml of supernatant was added to 10 ml of scintillation cocktail and the radioactivity assayed on a Beckman liquid scintillation counter. For each assay, background radioactivity was determined by sham incubation with dH_2O replacing blood (to assess the condition of the radio-substrate). The total amount of radioactive isotope used in each assay was also determined.

Normal control ChE activity was determined for each animal prior to drug exposure. This activity was designated as 100% activity for that animal. Relative ChE inhibition PI was plotted for each animal against its own control value.

We emphasize that enzyme inhibition levels were obtained at the prescribed intervals for <u>all</u> rats examined ultrastructurally and for all of the rats examined physiologically during Year II. Over 2,900 enzyme assays have been completed. Thus, enzyme histories are available for <u>each animal</u>, permitting direct correlation of altered ultrastructure with ChE enzyme inhibition.

10. PHYSIOLOGY

In vivo twitch tensions were recorded from EDL muscles in rats to determine the effects of acute and subacute physostigmine administration. During Year II, spinal block with lidocaine was used for all anesthesias. For the spinal block procedure, the rat initially was anesthetized with ether and a cannula inserted into the spinal canal at the L6 level. Sufficient 2% lidocaine was injected to maintain anesthesia in the caudal half of the body for about 25-30 minutes without producing generalized effects (decreased alertness, etc.). The rats were allowed to recover 1-2 hours from the ether anesthesia prior to recording of twitch tensions.

After removal of the overlying tibialis anterior muscle, the distal tendon of the EDL was dissected free, cut, and fastened to a Grass FT03C force displacement transducer. Resting tension was adjusted to 4 g. The peroneal nerve was isolated, cut, and placed on a bipolar stimulating electrode. The rat's paw and femur were clamped in a stereotaxic apparatus. During spinal blockade, the (alert) animal was immobilized in a restraining jacket. Exposed muscle and nerve were kept moist and the rat's body temperature maintained with a heating pad and radiant heat source. Twitch responses were recorded on a Graphtec WR3101 chart recorder and/or a Vetter Model D instrumentation tape recorder for detailed analysis.

Supramaximal (5T) stimulus pulses of 0.1 ms duration were delivered throughout the experiment at 0.1 hz except for special stimulation experiments designed to mimic the range of normal neuromuscular activity. 20 hz stimulus trains of 10 s duration were delivered at the beginning and end of recording sessions, to assess the ability of EDL to sustain high frequency contractions. For the high frequency stimulation experiments (to assess muscle use as well as the effects of stress), 20, 40 or 80 hz stimulus trains were delivered every 10 seconds throughout recording. Train durations were 3 or 6 seconds.

Acute physostigmine doses of 0.1 to 0.3 LD_{50} were injected subcutaneously in the rostral midback region for the "induced neuromuscular activity" experiments. For subacute and recovery experiments, Alzet pumps (if still in place) were removed under ether anesthesia prior to insertion of the spinal cannula and approximately 1½-2½ hours before recording was begun. Blood samples for ChE-enzyme inhibition analysis were taken at appropriate intervals.

F. RESULTS

1. BEHAVIORAL RESPONSES TO ACUTE PHYSOSTIGMINE

a. Initial Responses to High and Low Doses

Within 3-10 minutes after injection of 0.8 to 2.67 LD_{50} physostigmine, all rats exhibited pronounced fasciculation and tremor of superficial muscles, frequent tail rigidity, heightened startle reflex, increased salivation, pronounced respiratory distress, and increased ocular secretions. Rats that did not survive the initial acute exposure were characterized by pronounced hyperexcitability, uncoordinated lunging and running motions, extensive piloerection, and extreme respiratory distress, leading to convulsions and death within 2-30 minutes. Animals surviving supralethal doses usually retired to a corner of the cage and engaged in minimal muscle activity. Rats exposed to lower doses (0.001-0.1 LD_{50}) exhibited no overt signs of drug intoxication after they were released from the restraint (or in the initial experiments, after they had recovered from ether anesthesia).

b. Recovery

After 30 minutes of exposure to high doses (0.8-1.1 LD_{50}), surviving rats had greatly diminished or nearly normal startle reflexes. By 1 hour PI, the rats were resuming normal neuromuscular activity (i.e., brief walking excursions, drinking, and, occasionally, feeding). Within 6-24 hours, surviving rats exposed to 0.8-1.1 LD_{50} had resumed (near) normal activity. Rats were weighed periodically and immediately before perfusion fixation. Normal weight gain was noted in all acute exposure groups.

c. Effect of Stress on LD_{50}

We previously reported that rats recently delivered to the Animal Care Facility were found to be much more susceptible to physostigmine than rats "acclimated" for 7-10 days. In non-acclimated rats, for example, approximately 70% (5/7) died at 0.5 mg/kg, whereas in acclimated rats, approximately 5% (1/19) died at a higher dose of 0.6 mg/kg. Since serum ChE inhibition levels were similar for acclimated and non-acclimated animals exposed to similar amounts of physostigmine, we attributed the increased susceptibility in the "non-acclimated" rats to hyperexcitability and increased respiratory and voluntary muscle activity under conditions of stress. This observation suggests that, for military personnel, voluntary muscle activity be minimized during initial exposure to anticholinesterase agents, (i.e., nerve agents). (See section **"Interpretations"**).

d. Subacute exposures using Alzet miniosmotic pumps.

Rats were lightly anesthetized and Alzet pumps were implanted in small cavities made by incision in the rostral midback region. (The cavities were expanded by blunt dissections.) The pumps were briefly pre-primed by placing them in rat Ringers solution for 30 minutes to one hour.

e. Behavioral Response to Subacute Exposure

Upon recovery from the ether (usually within 10 minutes), the animals showed severe reaction only in the high dose exposure experiments. Signs of drug toxicity included prolonged respiratory distress, muscle fasciculations, bloody tears and muscle spasms. To minimize stress-induced damage during the initial exposure period, the room lights were darkened and the animals left alone and undisturbed. At 24 hrs, when the second blood sample was taken, the high dose animals remained hypersensitive and vocal, but showed no respiratory distress or bloody tears. By that time, they were also mobile and were eating and drinking. During the next 13 days, the only obvious effects of the drug were a gradual weight loss and a continuing "malaise" (i. e., animals were subdued with evident intentional reduction of muscle activity). Other long-term behavioral and gross physiological effects were not detectable in high dose animals 7 days or more after Alzet pump implantation. In contrast, the low dose and control animals steadily gained weight and showed no signs of drug toxicity.

Interestingly, during the stress of prolonged physiological monitoring, some animals in the high-dose group developed obvious signs of physostigmine toxicity after about 1 hr of recording. (See Section F.4c) Low dose animals showed no altered behavioral response at any time during physiological recording.

2. WHOLE BLOOD ChE LEVELS

a. Acute Exposures to Physostigmine

Blood cholinesterase enzyme assays have been completed on more than 270 animals (2,900 separate assays). Blood samples were taken immediately before injection of physostigmine and at the following intervals PI: 30 minutes and 1, 7, 14, 28, and 56 days (or until the animals were prepared by perfusion fixation).

In Year 1, we reported stability of samples for enzyme analyses, as well as high reproducibility between two samples taken simultaneously from the same animal.

In all cases, normal blood cholinesterase enzyme activity was measured for each animal just prior to exposure to physostigmine. The "pre-drug" enzyme activity was assigned the value of 100%. At 30 min after exposure to physostigmine, inhibition of blood cholinesterase (30 minutes PI) was linearly proportional to the log of the physostigmine dose between 0.01-0.8 LD_{50} (0.0075 mg to 0.6 mg/kg). At 30 minutes PI, the following blood ChE enzyme inhibitions were measured:

$$0.001\ LD_{50} = 28\% \pm 15\%$$
$$0.01\ LD_{50} = 33\% \pm 7\%$$
$$0.1\ LD_{50} = 67\% \pm 7\%$$
$$0.8\ LD_{50} = 89\% \pm 3\%$$

In the First Annual Report, we also showed that the blood cholinesterase activity of animals not exposed to physostigmine (i.e., the "controls") fluctuated substantially from day to day. Moreover, in our First Annual Report we cautioned that these data for blood ChE inhibitions, especially for the very low dose exposures, may not correlate with actual endplate AChE inhibitions (see Interpretations).

b. Subacute Exposure

For subacute exposure experiments, during Year I, the doses and implantation regimens required to obtain 30% and 80% blood cholinesterase enzyme inhibition were determined. Exposure levels of 0.25 mg/kg and 25 mg/kg delivered at a rate of 2.5 µl/hr yielded serum ChE inhibitions of 30-50% and 70-80%, respectively. These values are equivalent to a delivery of 0.003 LD_{50}/hr and 0.3 LD_{50}/hr. Nevertheless, this 100-fold difference in drug delivery with only a 2-3 fold difference in blood ChE inhibition, is consistent with data from acute exposure experiments.

For the high dose group, blood ChE inhibitions of 70-80% were maintained for 14 days (in one case up to 21 days, Fig. 1). After the Alzet minipumps were removed, ChE enzyme levels returned to normal or above normal and remained at that level for the entire 28 day recovery period. In the high dose group of animals, some animals showed far less inhibition (Table A, Appendix).

For the "low dose" group (Fig. 2), approximately 50% ChE inhibitions were obtained in most animals and were maintained for up to 14 days. (A second "anomalous" group showed no apparent enzyme inhibition at the same dose level.) This may indicate a) pump failure in those animals, or b) low initial "control" values. (The latter explanation is favored because those few

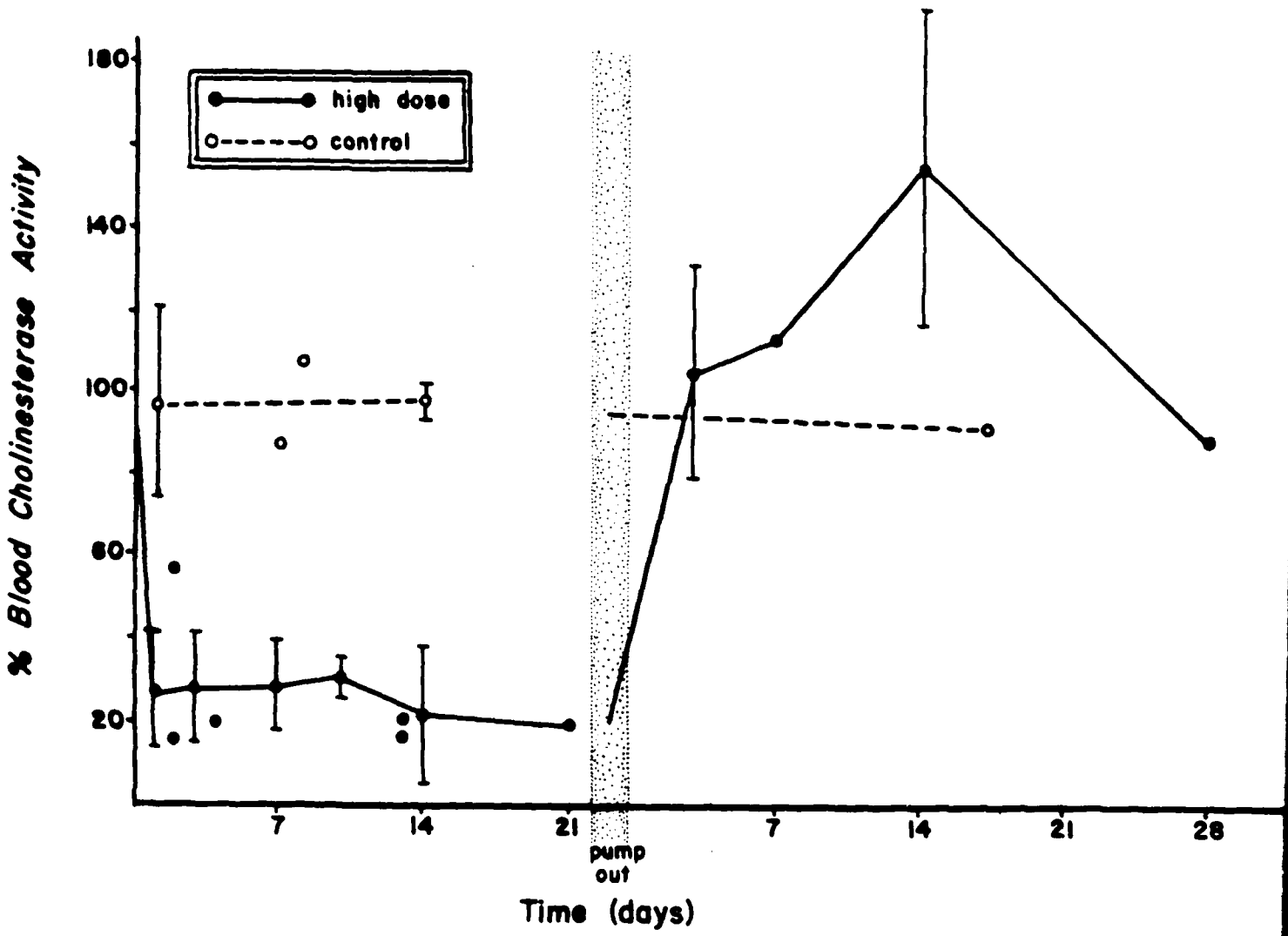


Figure 1. Blood cholinesterase activity as a function of subacute exposure to 25 mg/ml delivered at a rate of 25µl/hr (high dose). The error bar represents one standard deviation. The return to normal ChE activity after Alzet pump removal is plotted after the vertical bar.

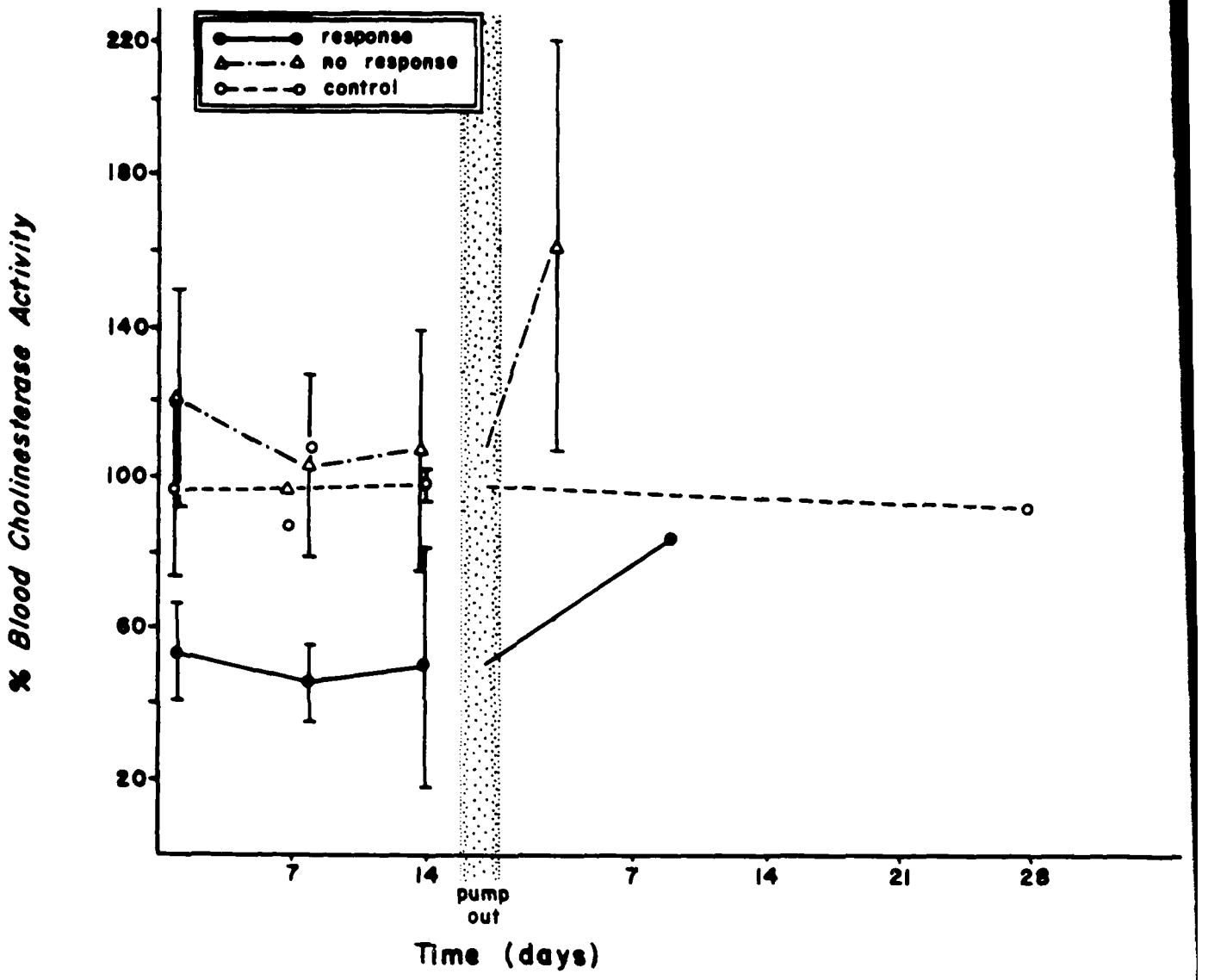


Figure 2. Blood cholinesterase activity as a function of subacute exposure to 0.25 mg/ml delivered at 25 µl/hr (low dose). The error bar represents one standard deviation. The ChE activity after Alzet pump removal is consistent with little or no long term effect on blood ChE.

animals responded to pump removal by a "rebound" of ChE level to about 160% of "normal". If that latter value is presumed to be the "true" "control" value, then all enzyme inhibitions were near the projected 30-50% level.) In addition, some animals developed thickened "capsules" around the pump implants. However, variation in blood ChE did not correlate with capsule formation. Regardless, both low dose groups ("ChEase responders" and "ChEase-non responders") were identical in their lack of ultrastructural alterations at all durations of exposure and in all "recovery" stages.

The implantation of "control" Alzet pumps (which contained buffer but not physostigmine) and their removal had no observable effect on blood ChE (Fig. 1, control).

Detailed data for ChE inhibitions showing the overall data spread are presented in in Section IX, Appendix, Tables A-C.

3. **TASK** I:a. DOES SUPERCONTRACTION OCCUR AFTER ACUTE HIGH DOSE EXPOSURE TO PHYSOSTIGMINE?
b.IF SO, IS SUPERCONTRACTION FIBER-TYPE SPECIFIC?

a. SUPERCONTRACTION OCCURS AT HIGH DOSES.

1) Light Microscopic Observations of Neuromuscular Junctions of Rats Exposed to High Dose of Physostigmine (Fig. 3) were reported in YEAR I. In the remainder of this study, only electron micrographs will be utilized because they are easier to obtain than light micrographs, exhibit fine structure even at the lowest available magnifications, and their interpretation is less subjective.

2) Ultrastructure of Neuromuscular Junctions of Rats Exposed to High Dose of Physostigmine (Acute and Subacute)

In Year I, we showed that the **diaphragm is the best model** for analyzing the immediate, life-threatening effects of physostigmine (i.e., blockage of respiration). Diaphragm fibers are consistently and grossly affected by physostigmine; soleus fibers somewhat less affected; and EDL fibers consistently the least affected. After a single high dose acute exposure to physostigmine (0.8 to 1.1 LD_{50}, 30 min elapsed time before perfusion fixation) all (10 out of 10) neuromuscular junctions of diaphragm myofibers (Fig. 3 and 4), including both fast twitch and slow twitch fibers exhibited characteristic signs of supercontraction, often extending up to one hundred microns outside the neuromuscular junction (Fig. 3). Likewise, in diaphragm myofibers from animals receiving 0.3 LD_{50} (75% inhibition), most fibers (60%) showed supercontraction of the endplate region, but restricted solely to the subjunctional sarcoplasm (Fig. 5). In these "suprathreshold" but sub-lethal doses, concentric zones reflecting a continuum of severely to slightly damaged mitochondria were observed in the subjunctional sarcoplasm (Fig. 4 and Fig. 5). Progressing from the junctional folds to undamaged regions, the sequence was "exploded," to "frothy," to "blistered" mitochondria, and at the margins of the supercontracted areas, mitochondria with excess numbers of electron dense "calcium phosphate granules". (We define "blistered" mitochondria as those that have smooth regular margins with one or a few scattered "lens-like" areas of partially clear mitochondrial matrix. "Frothy" mitochondria are those with numerous "blisters" and, perhaps, occasional lateral swellings that distend the surface contour. "Exploded" mitochondria are grossly distended, often with irregular surface contours and separations of inner and outer mitochondrial membranes. In the extreme case, "exploded" mitochondria have obvious discontinuities in their external membranes.)

Supercontraction was never observed in diaphragm myofibers at 0.1 LD_{50} (67% inhibiton) in unstressed rats. Thus, it is clear that threshold for supercontraction in diapragm is attained at about 0.2 to 0.3 LD_{50} or 10%-75% serum ChE inhibition.

In regions of maximum supercontraction in the diaphragm and soleus muscles exposed to 0.8 LD_{50} (Fig. 6), partial or complete destruction of the myofibrillar apparatus was observed, including the disappearance of Z bands and the dissolution of recognizable sarcomeres. Supercontraction was observed in only a few fibers (<20%) in EDL at ½hr PI (Fig. 6.). Since the EDL consists almost exclusively of fast twitch fibers while the soleus is composed almost entirely of slow twitch fibers, one might ask "Are the differences observed in supercontraction due to fiber type?"

b. THE CYTOPATOLOGICAL EFFECTS ARE NOT FIBER-TYPE SPECIFIC

The diaphragm is a mixed muscle containing fast twitch, intermediate, and slow twitch myofibers. All three fiber types in the diaphragm were equally severely affected by supralethal doses. Thus, we concluded in the first Annual Report that the damaging effects are not fiber-type specific, but instead are related to the obligatory muscle use patterns of the diaphragm. In this second Annual Report, we continue to confirm those data and interpretations and now call attention to a characteristic radiating pattern of damage which appears to reflect the relative contributions of ChE inhibition and muscle use on the expression of the "threshold" for cytopathological alterations. (See also Fig. 3, at 0.3 LD_{50} and more detailed description in Section F.5.)

Figure 3. Side-by-side comparison of light and electron micrographs showing the effects of 30 minutes of acute high-dose exposure to physostigmine (0.8 LD_{50}) on neuromuscular junctions of diaphragm (Figs. 3a,b) and soleus muscle (Figs. 3b,d). Supercontraction and disruption of myofibrils in the subjunctional sarcoplasm of diaphragm and soleus fibers are evident, as is mitochodrial swelling (arrowheads). However, at light microscopic magnification and resolution, endplates are difficult to find/identify, and resolution of pathological detail is not always possible. On the other hand, in even the lowest magnification electron micrographs (Figs. 3b,d), effects of physostigmine are clearly resolved. Comparison of Fig. 3b and Fig. 3d reveals that the extent of supercontraction and disruption of myofibrils is much greater in diaphragm myofibers (up to 50-100μm from the NMJ) than in soleus fibers (subjunctional only).

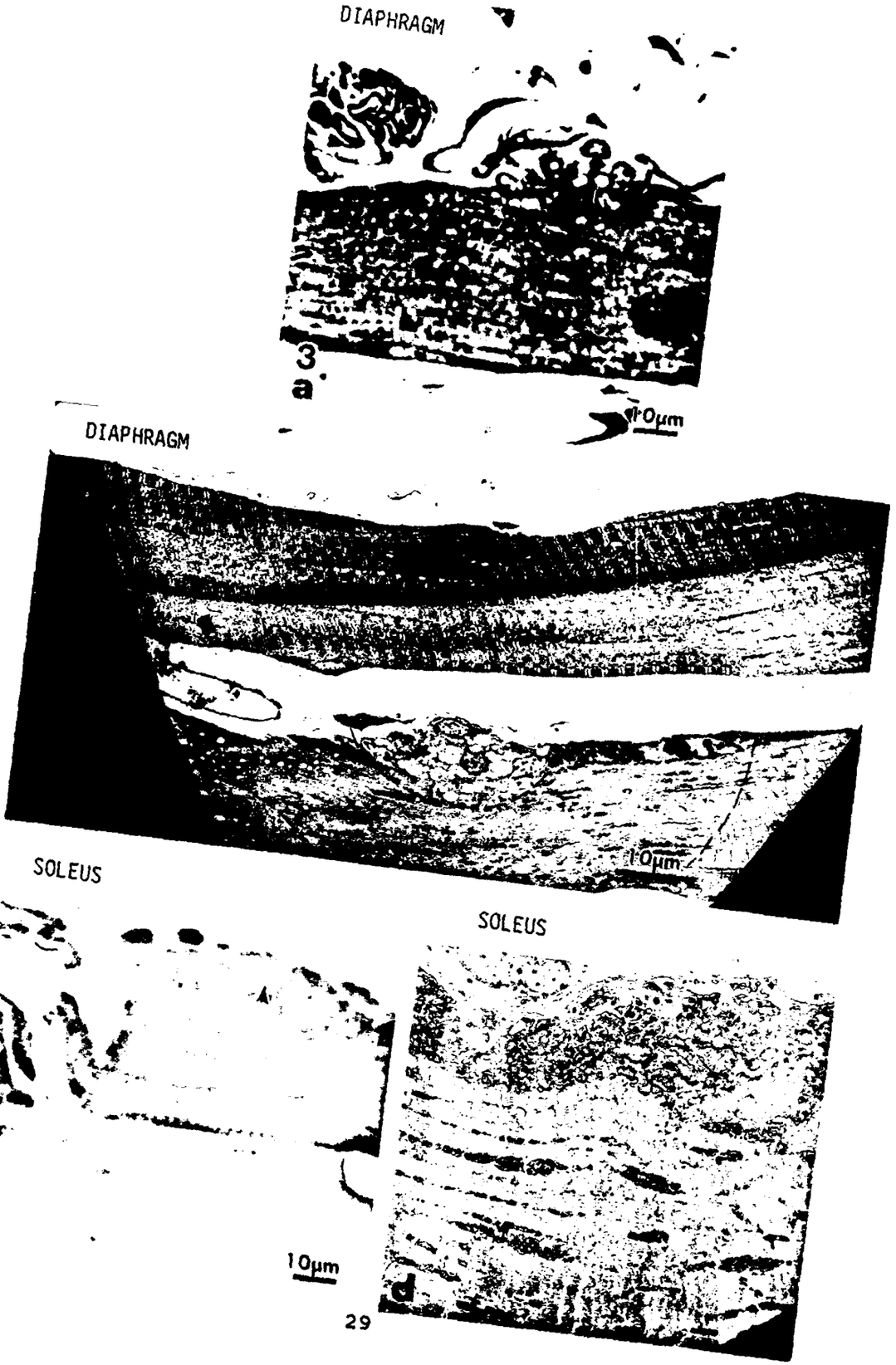

DIAPHRAGM
3.
a
10μm
DIAPHRAGM
10μm
SOLEUS
SOLEUS
c
10μm
d

Figure 4. Effects of acute high dose (0.8 LD_{50}) of physostigmine on neuromuscular junctions of rat diaphragm 30 minutes post injection (PI). In regions of severe supercontraction, complete destruction of the myofibrillar apparatus was observed. Z bands had disappeared and sarcomeres were no longer recognizable. A continuum of severely to slightly damaged mitochondria ("exploded" to "frothy" to "blistered") was observed in the subjunctional sarcoplasm in all diaphragm myofibers from the high dose exposure groupl (Figure 4 is a higher magnification view of the endplate region in Figure 3b.)

HIGH DOSE

DIAPHRAGM

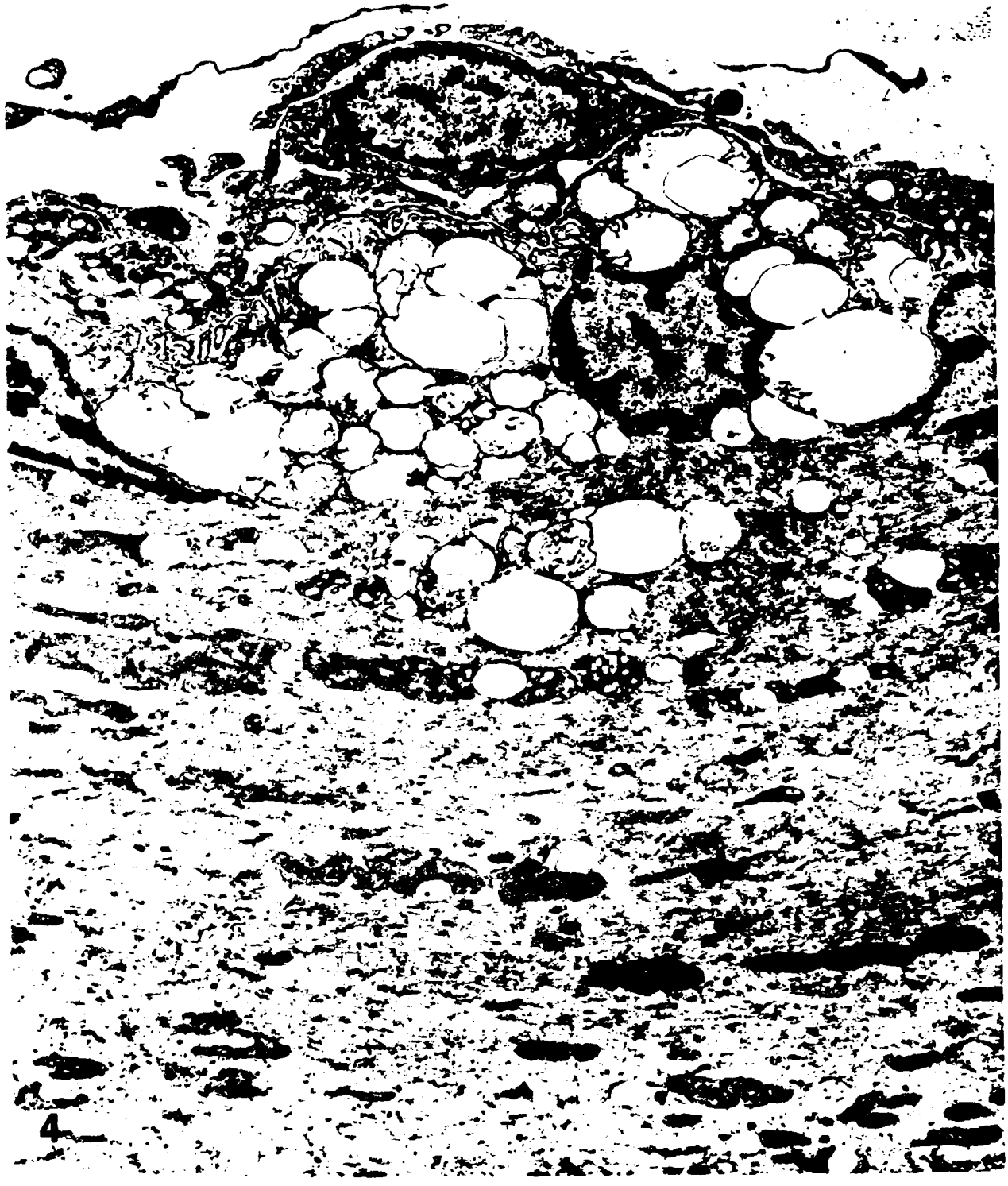


Figure 5. Effects of a 0.3 LD_{50} dose of physostigmine (75% serum ChE inhibition) on neuromuscular junctions of diaphragm muscles. At this dose, about 60-70% of neuromuscular junctions examined exhibited partial supercontraction. As indicated by the concentric dotted lines, (see also Fig. 3b), a continuum of mitochondrial alterations (from "exploded" to "blistered", with the most severely affected mitochondria nearest the junctional folds, and those with decreasing effect observed at greater distances from the junctional folds).

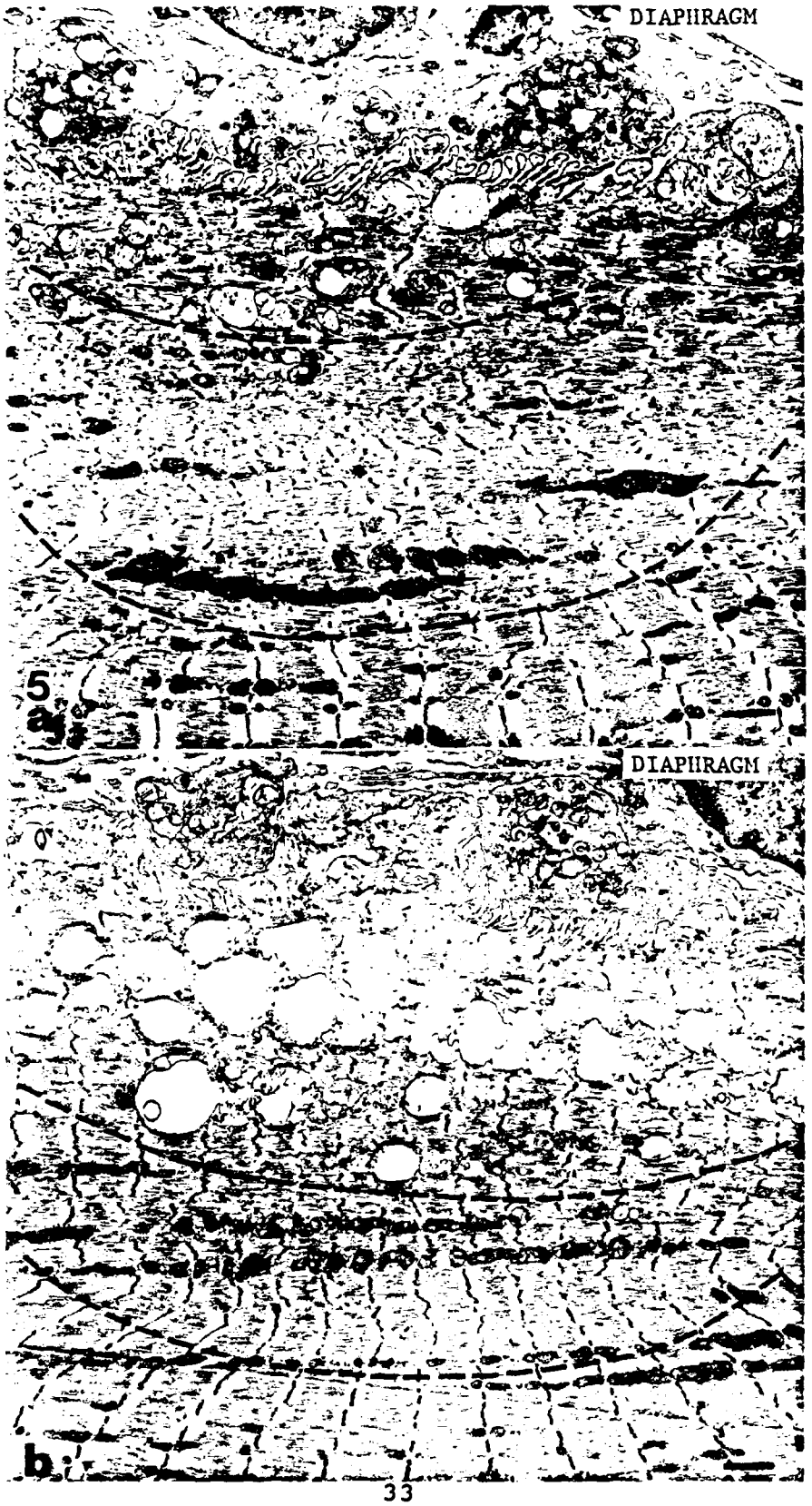
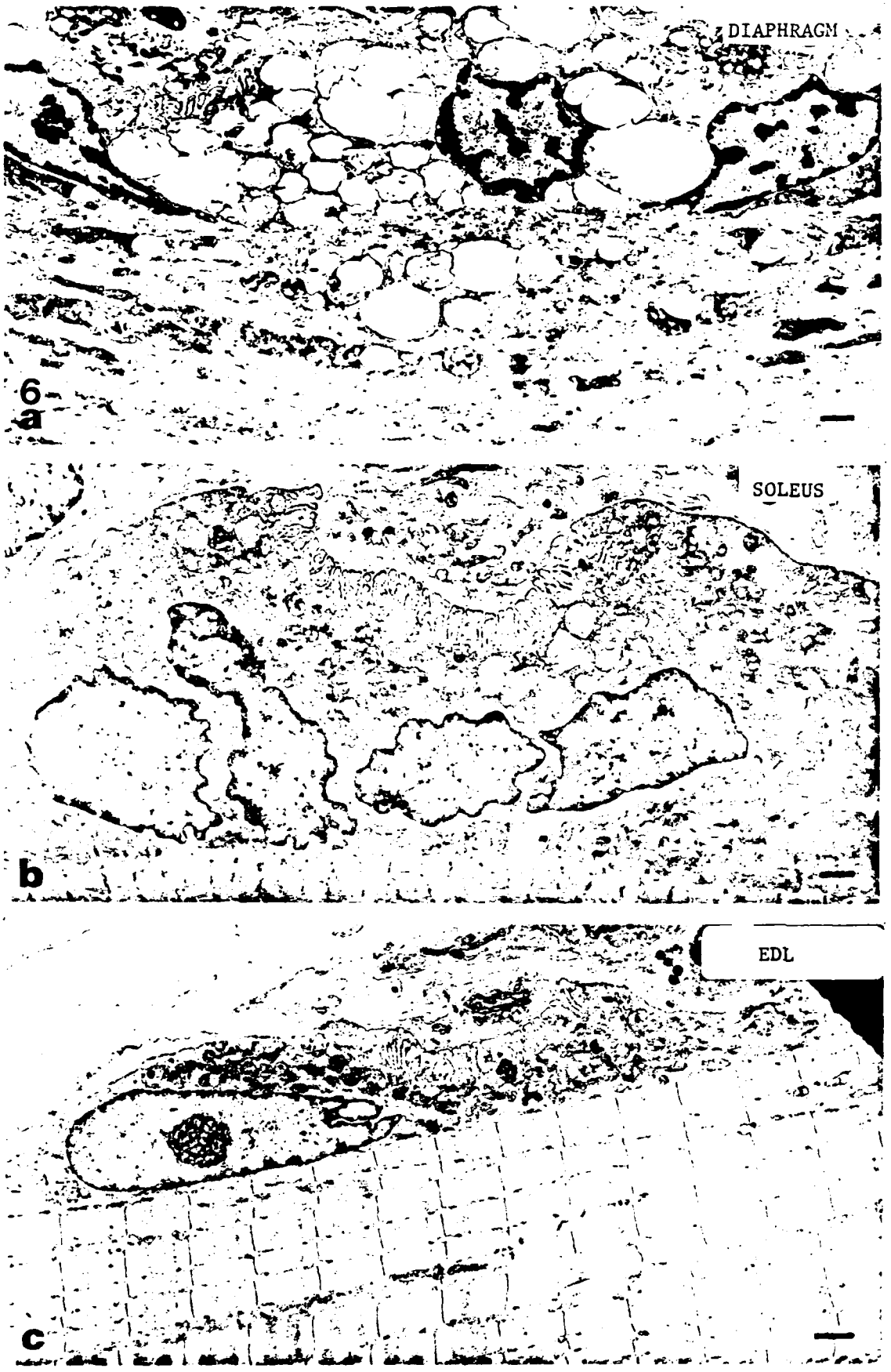

DIAPHRAGM
5
DIAPHRAGM
b

Figure 6. Comparison of the effects of acute high doses of physostigmine (0.8-1.1 LD_{50}) on neuromuscular junction of diaphragm (Fig. 6a), soleus (Fig. 6b), and EDL muscles (Fig. 6c) 30 minutes PI. All neuromuscular junctions of the diaphragm and soleus myofibers exhibited supercontraction of sarcomeres in the subjunctional sarcoplasm. In contrast, EDL muscles from the same rats were much less affected at 30 minutes and exhibited nearly normal ultrastructure. Minor blistering of subjunctional mitochondria was observed in many EDL myofibers.


DIAPHRAGM
6
a
SOLEUS
b
EDL
c

4. **TASK II:** SUBACUTE EXPOSURE TO PHYSOSTIGMINE

a. DOES CONSTANT RATE OF PHYSOSTIGMINE INFUSION YIELD A CONSTANT LEVEL OF ChE INHIBITION?

b. IF SO, DOES SUPERCONTRACTION OCCUR DURING CHRONIC ADMINISTRATION OF PHYSOSTIGMINE?

c. WHAT ARE THE PHYSIOLOGICAL EFFECTS OF SUBACUTE EXPOSURE?

a. A CONSTANT RATE OF PHYSOSTIGMINE INFUSION WAS FOUND TO YIELD A RELATIVELY CONSTANT RATE OF SERUM ChE INHIBITION.

Whole blood ChE levels were monitored for rats implanted with Alzet pumps loaded with physostigmine at a concentration of 0.001 mg/ml to 1 mg/ml and delivered at a rate of 2.5 µl/hour. After initial difficulties related to prolonged "pre-priming" of pumps (See 1st Annual Report), the desired levels of 30% and 80% ChE inhibition were obtained within 1 hr and were maintained for up to 14 days of subacute exposure. (Final concentrations of 0.25 mg/ml and 25 mg/ml were delivered at a rate of 2.5 2.5 µl/hr.) Thus, the acetic acid carrier solutions proved effective in maintaining physostigmine activity/ChE inhibition (30% and 80% inhibition) for the entire exposure period. Confirmation of potency of the residual contents was also performed as described in "Methods". Thus, goal IIA was attained and the subacute exposure experiments were completed on schedule.

b. SUPERCONTRACTION RARELY OCCURS DURING SUBACUTE EXPOSURE TO A "THRESHOLD" DOSE.

1) High Dose.

Rats were implanted with Alzet pumps which maintained enzyme inhibitions of 80-90% (high dose) and 30-50% (low dose) for 3, 7, and 14 days. By comparison to acute exposures in which 80-90% ChE inhibition was found to be at or just above the "threshold" for producing myofiber damage in the diaphragm, hign dose subacute exposures with sustained 80% inhibition produced few changes in diaphragm, soleus, or EDL myofibers after 3 days (Fig. 7. "SH3" designates Subacute Highdose-3 day exposure.) Most diaphragm and EDL muscles were also found normal at 7 days (Fig. 8). However, a few soleus muscles had damaged myofibers in the subjunctional sarcoplasm (Fig. 8b). [The source of this temporal and pharmacological variability has not yet been determined, but possible sources include a) limited endplate sampling size inherent to TEM, b) variations in enzyme inhibition in different animals, c) differential susceptibility of fast twitch vs. slow twitch fiber combined with different use patterns, and d) different muscle use patterns in different rats.]

At 14 days (Fig. 9), the subjunctional sarcomeres of all muscles appeared normal. However, the subjunctional cytoplasm of diaphragm and soleus (but not EDL) myofibers had become hypertrophied or "edematous" and contained swollen nuclei with compacted (thin) peripheral heterochromatin. On the other hand, "frothy" or swollen mitochondria were not observed. The source of the subjunctional hypertrophy/edema may reflect either response to prolonged endplate depolarization and associated ion and water influx, to increase in biosynthetic activity or to unknown factors. However, these data indicate near threshold expression of toxic effects, as well as expression of cumulative effects of the drug over the 14 day exposure period.

2) Sub-acute low dose.

At low dose (30-50% ChE inhibition), no supercontractions or other evidence for damage were detected in any fibers at any exposure interval (Figs. 10a-c). [Since diaphragm myofibers in all other exposure groups were consistently most affected, and since no cytopathological alterations were noted in any rat exposed to subacute low dose at 3, 7 or 14 days, we have shown only diaphragm myofibers as being most representative.] From these data we conclude that during subacute exposure at moderate doses (30-50% inhibition), supercontraction does not occur. Thus, extended low to moderate dose subacute exposure to physostigmine seems to be well tolerated by both stressed and unstressed rats.

Figure 7. A subcute high dose 3 day exposure (designated SH3 on the micrograph label) to physostigmine (80% serum ChE inhibition) produced few detectable changes in neruomuscular junctions of diaphragm (Fig. 7a), soleus (Fig. 7b), and EDL (Fig. 7c) myofibers. Myofibrils just beneath the junctional folds were slightly misaligned with slight skewing of Z bands, which was obviously only in soleus myofibers. In the soleus muscle (Fig. 7d), the subjunctional finger-like projections and probably tangentially sectioned junctional folds.


DIAPHRAGM
SH3
7
a
SOLEUS
b
EDL
c

Figure 8. Effects of subacute high dose 7 day exposure (SH7) on neuromuscular junctions of diaphragm (Fig. 8a), soleus (Fig. 8b), and EDL (Fig. 8c) muscles of. Mitochondria and nerve terminals in all myofibers appeared normal. Myofibers of diaphragm and EDL muscles appeared normal. However, damaged myofibrils with "zig-zag" Z bands and dissolution of recognizable sarcomeres were found in several fibers from the soleus muscle of one animal. Thus, variability in effect was observed.

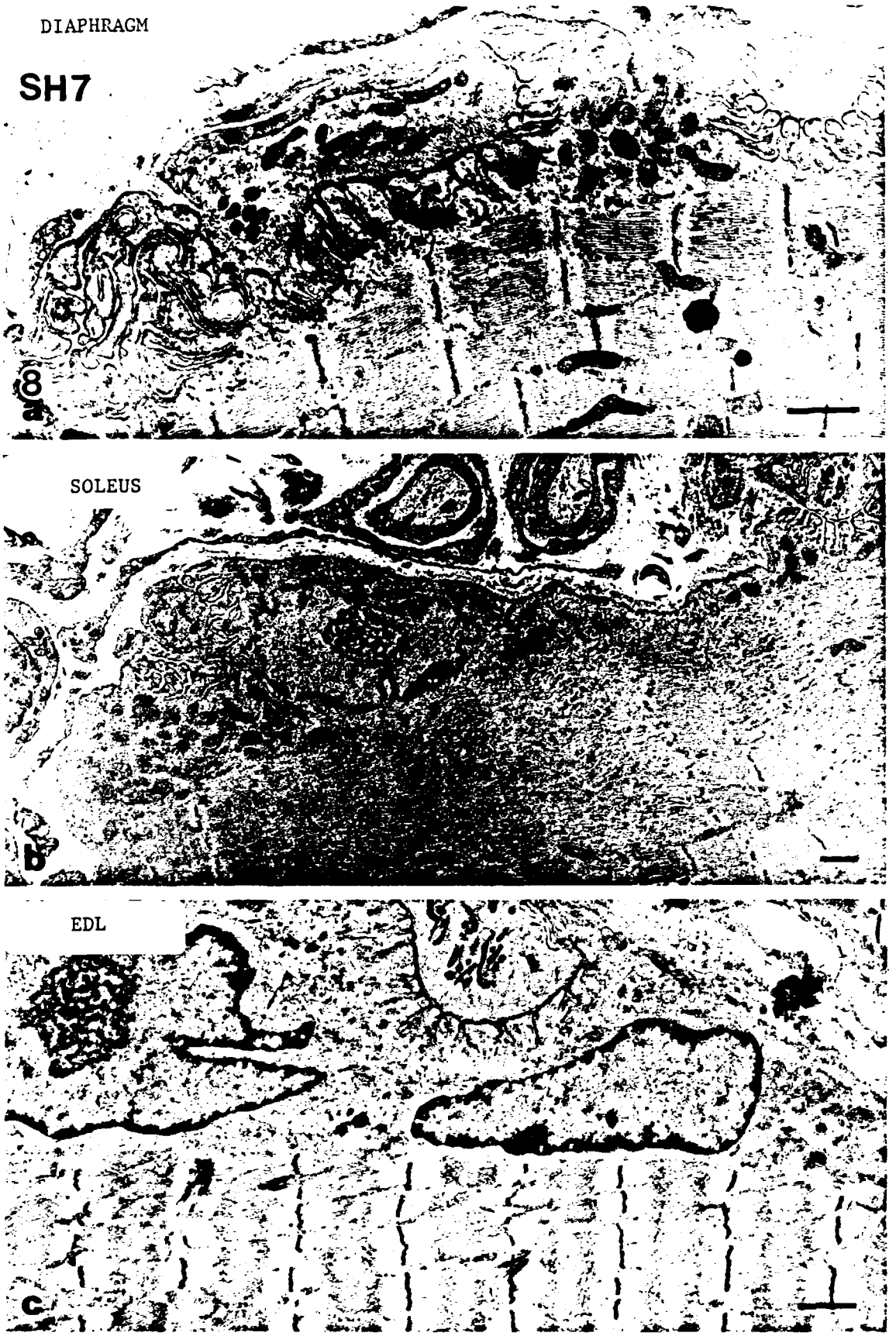

DIAPHRAGM
SH7
SOLEUS
EDL

Figure 9. Effects of a subacute high-dose of physostigmine (80% serum ChE inhibition) on neuromuscular junctions of diaphragm (Fig. 9a), soleus (Fig. 9b), and EDL (Fig. 9c) after 14 days of subacute exposure (SH14). Myofibers, mitochondria, and nerve terminals in all muscles appeared normal. However, the volume of subjunctional cytoplasm was increased in the diaphragm and soleus myofibers (i.e., appeared "edematous"). Nuclei in the diaphragm were swollen, with heterochromatin condensed tightly against the inner nuclear membrane.

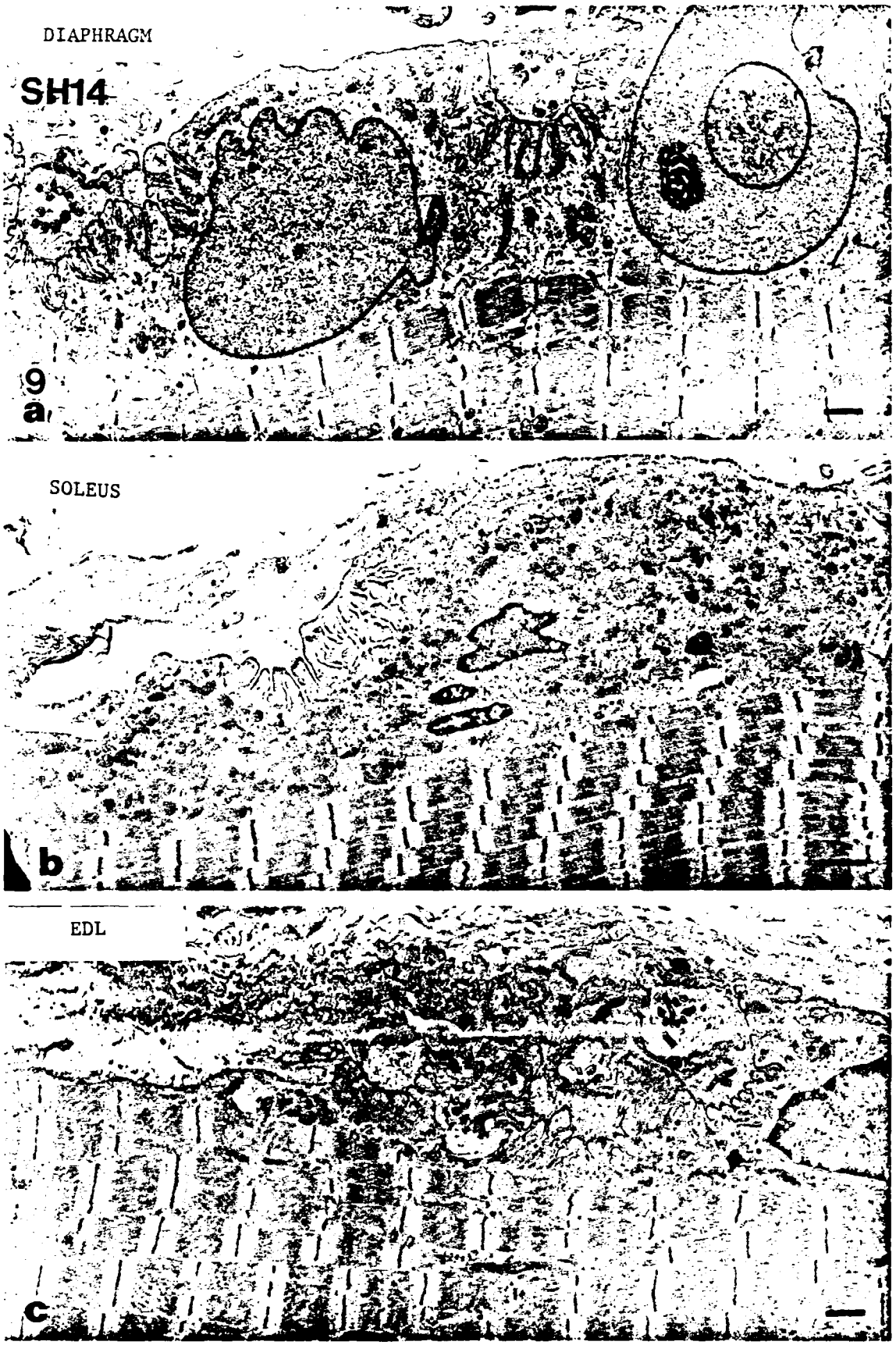
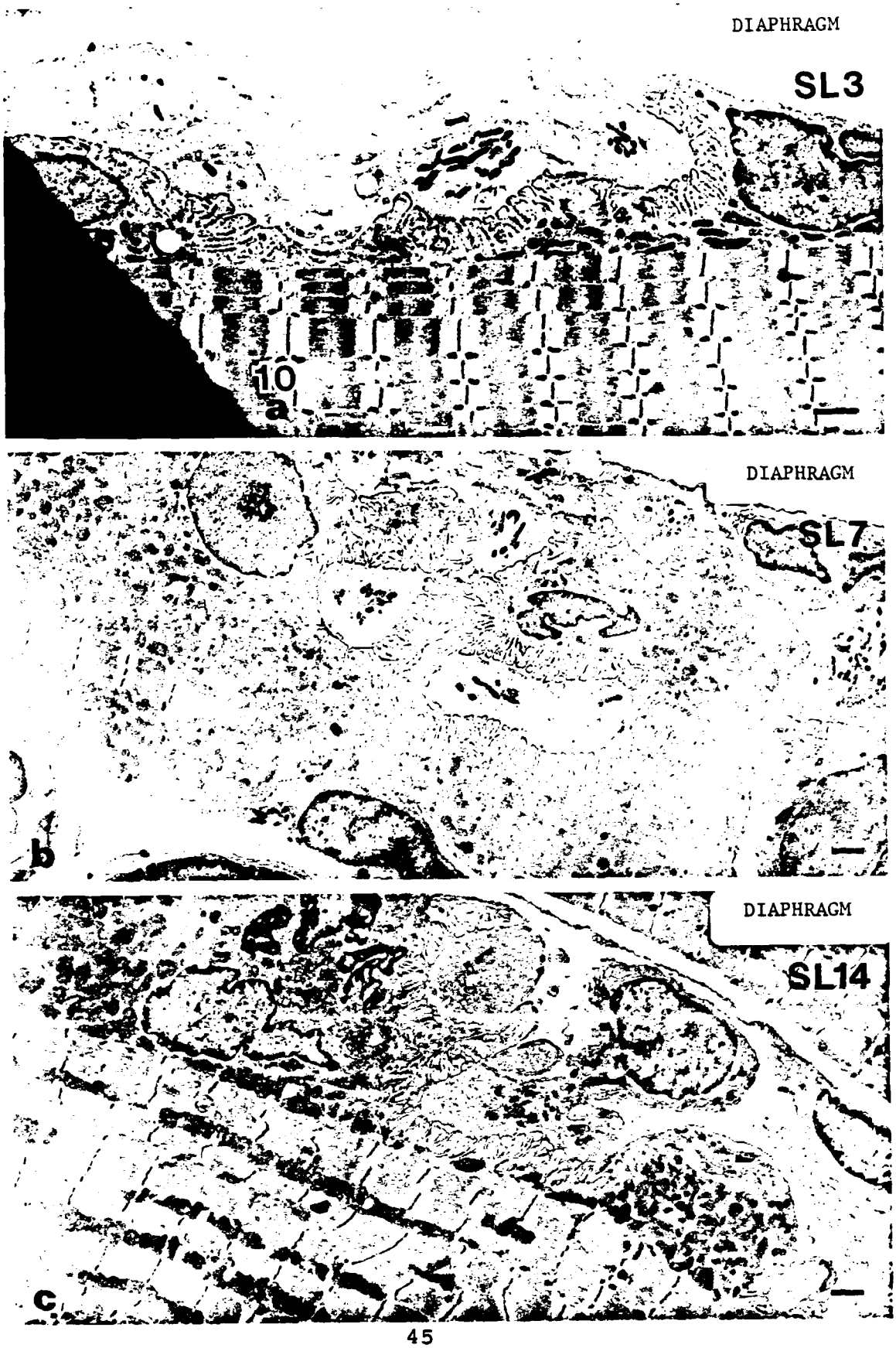

DIAPHRAGM
SH14
9
a
SOLEUS
b
EDL
c

Figure 10. Effects of a subacute low dose of physostigmine (30% serum ChE inhibition) on neuromuscular junctions of diaphragm after continuous 3 day (Fig. 10a), 7 day (Fig. 10b) and 14 day (Fig. 10c) exposures (SL3, SL7, SL14). Myofibers, mitochondria, and nerve terminals appeared normal.


DIAPHRAGM
SL3
10
a
DIAPHRAGM
SL7
b
DIAPHRAGM
SL14
c

c. PHYSIOLOGICAL EFFECTS OF SUBACUTE EXPOSURE ARE MINIMAL.

Physiological effects of subacute physostigmine administration on in vivo muscle contractility have been obtained from 22 rats. Ten animals had either high or low dose Alzet pumps inserted for 3, 7 or 14 days before testing. An additional 10 rats had high or low dose pumps implanted for 14 days followed by 3, 7 or 14 days recovery after pump removal ("subacute/recovery"). Two additional rats were implanted with Alzet pumps containing acetic acid carrier solution and were designated as "controls".

During surgical preparation and the first hour of recording, none of the rats from either the high or low dose subacute exposure groups showed any overt signs of physostigmine intoxication (i.e., no muscular fasciculations, respiratory difficulty, chromodacryorrhea, etc.). Likewise, neither of 2 high dose animals that were tested with pumps still in place initially showed any signs of physostigmine toxicity, although toxic symptoms did appear later (described more fully below).

Average EDL twitch tensions, measured during the first 10 minutes of recording, are summarized in **Table 2.** The overall average twitch tension for all subacute exposure regimens was 28 $\pm$4 g, (i.e., not significantly different from averages for any of the subgroups in Table 2 or from the normal population value of 30 g $\pm$7 g obtained during the acute exposure experiments.)

Table 2. Average in vivo EDL twitch tension for subacute administration of physostigmine.

Treatment	Twitch Tension (g)
High dose/No recovery	28 $\pm$ 2 (N=10)
High dose/Recovery	26 $\pm$ 8 (N=5)
Low dose/No recovery	28 $\pm$ 9 (N=3)
Low dose/Recovery (3-14 days)	29 $\pm$ 6 (N=5)
Control Population	30 $\pm$ 7 (N=28)

The ability of EDL to sustain contraction with 20 hz stimulation was used as an indicator of possible physostigmine-induced damage to the contractile properties or to the motor endplate. As in the First Annual Report, data are expressed as ratios of the maximum tension developed at the start of a 20 Hz stimulus train divided by the tension at the end of the 10 second train. A larger "tension ratio" indicates decreased ability to sustain contraction.

Tension ratios measured during the first 10-15 minutes of recording were not significantly different ($P<.01$) from controls for any of the rats with high dose Alzet pumps (recovery or non-recovery). A control value of 1.1 ± 0.2 was obtained during the acute studies prior to injection of physostigmine. Average tension ratios for subacute (Alzet pump) rats were 1.0 ± 0.2 for high dose/non-recovery and 0.9 ± 0.2 for high dose/recovery. (Sizes of low dose groups were too small to compile a statistically significant sample, but average tension ratios were measured at 1.0 and 1.1 for recovery and non-recovery, respectively.)

A possible indication of physostigmine-induced damage was demonstrated by comparing the change in tension ratios during prolonged EDL stimulation. An increase in tension ratio after 15-30 minutes of stimulation at 0.1 hz suggests that the EDL fatigues more easily than normal and loses some ability to sustain a contraction. The results of such a comparison are given in **Figure 11**, where days with pumps implanted and subsequent recovery are plotted versus the change in tension ratio. For both high and low doses, a general trend toward larger increases in tension ratio (and thus, greater fatigability) after 15-30 minutes stimulation is demonstrated after 14 days of pump implantation. This was followed by a gradual decrease in tension ratio ("recovery") following pump removal. These data should be interpreted with caution, however, because of the small sample size (each group represents only 1 to 3 rats).

Blood samples taken either immediately or within 15 minutes after the start of recording gave enzyme inhibition levels of 59% to 88% (average 74%) for high dose/non-recovery rats and 0% for high dose/recovery animals (i.e., complete recovery of normal enzyme activity). Moreover, when these blood samples were taken, neither EDL twitch potentiation nor other visible signs of physostigmine toxicity were detectable.

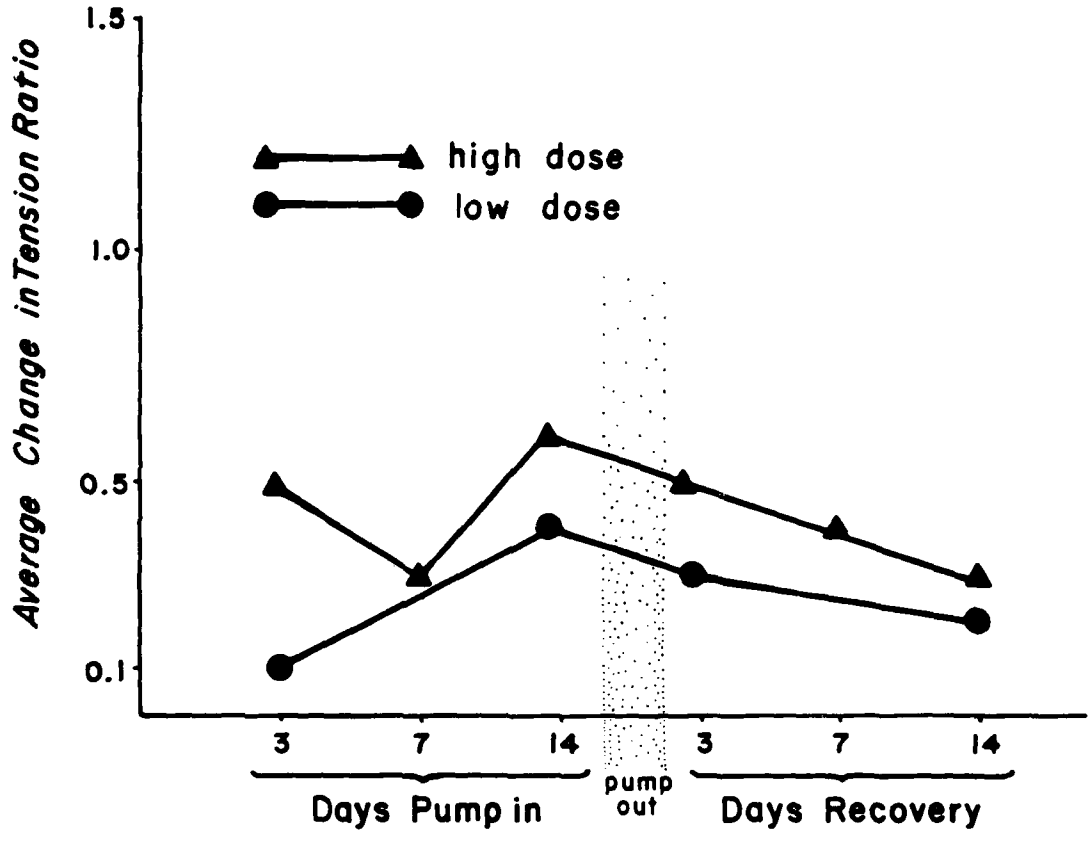


Figure 11. Ability of EDL to sustain 20 hz contraction during and subacute physostigmine administration. Tension ratio is force generated by the EDL at the beginning of a 20 hz stimulation train divided by force at the end of the 10 second train, higher tension ratio indicating decreased ablity to sustain contraction. Values plotted represent the change in tension ratio after 15 to 30 minutes stimulation at 0.1 hz.

5. THE EFFECTS OF MUSCLE USE ON THRESHOLD OF TOXICITY:

a. Physiology

1) Induced toxicity during electrophysiological monitoring.

Two animals implanted for 14 days with high dose pumps were tested with pumps still in place. During surgical preparation and the first hour of recording, neither animal showed physiological signs of physostigmine intoxication. Blood samples taken at this time gave cholinesterase inhibition levels of 80 and 88%. After about one hour of recording, both animals developed facial fasciculations and chromodacryorrhea as well as potentiation of EDL twitch tension (maxima of 130% and 230% of normal). Data available for one of these rats showed a blood cholinesterase inhibition of 87% during the physostigmine response. The cause of the onset of toxic symptoms one hour into the recording session is not known, but prolonged stress and/or increased respiratory load are considered to be possible causes.

2) Muscle Use

Experiments to assess the effect of muscle use on physostigmine-induced pathology have employed low to high frquency stimulation of the common peroneal nerve (which innervates EDL and soleus) to mimic normal muscle use patterns. Stimulation patterns have included 20 hz trains of 3 or 5 sec duration, 40 hz trains of 3 sec duration, and 80 hz trains of 6 sec duration, delivered from 10s to 2½ min apart. Subcutaneous doses of physostigmine at a near threshold level (0.3 LD_{50}) produced cholinesterase inhibition levels of 78% to 89%.

For the experiments using 20 hz stimulus trains at various intervals, EDL muscles showed a slightly increased tension ratio (i.e., decreased ability to sustain contraction) during the physostigmine reaction. However, tension ratios returned to normal as the rats recovered. Controls (no physostigmine) with 20 hz trains maintained approximately the same tension ratios throughout an hour of recording. Rats stimulated with 40 hz and 80 hz trains responded to physostigmine injection with substantially increased tension ratios when tested at the same stimulation frequencies, and even larger tension ratios (i.e., decreased ability to sustain contraction) when tested with 20 hz trains. These same animals were then prepared for ultrastructural analysis

b. Ultrastructure

In rats exposed to 0.3 LD_{50} physostigmine, most diaphragm myofibers (constantly used in breathing) had subjunctional sarcomeres that were supercontracted (Fig. 12a). In contrast, in unstimulated myofibers of soleus and EDL muscles from the same rats (not shown), and in EDL myofibers stimulated at 20 hz (Fig. 12c), no ultrastructural alterations were observed. On the other hand, soleus fibers stimulated at 20 hz had a few blistered and (occasionally) frothy subjunctional mitochondria (Fig. 12b). At 40 hz and 80 hz, EDL myofibers exhibited stepwise increases in swelling of mitochondria, endoplasmic reticulum, golgi cisternae, and nuclear membranes, but did not exhibit supercontraction (Figs. 13c & 14c). Moreover, swelling of presynaptic mitochondria was consistently observed in these heavily stimulated EDL endplates (Fig. 14c) but not in equally stimulated soleus endplates (Fig. 14b.) (Whether these presynaptic changes are due to sustained hyperactivity of the nerve terminal or to secondary drug effect cannot be ascertained from this experiment.) Interestingly, greater damage to soleus fibers occurred at a stimulation frequency of 20 Hz (Fig. 12) than at 40 Hz or above (Figs. 13 & 14). This may be due to failure of neuromuscular transmission in this slow twitch, tonically activated muscle when stimulated at a frequency above the tetanic fusion frequency. Finally, we note that the appearance of damage only in myofibers that had been stimulated at or above normal use patterns suggests that the threshold for physostigmine-induced damage is altered substantially by muscle use. At or just below normal threshold levels, the extent of damage was found to be proportional to stimulation frequency.

The physiological and ultrastructural analyses of these experiments are incomplete at present. Physostigmine doses producing lower enzyme inhibition levels must be tested before conclusions can be made concerning the interaction of physostigmine dose, stress, and muscle use in the production of muscle pathology. A Supplemental Request has been submitted to investigate further the phenomenon of stimulation-induced damage to myofiber ultrastructure.

6. **TASK III:** DOSE RESPONSE RELATIONSHIPS:
   a. FOR ACUTE ADMINISTRATION,
   b. FOR ACUTE-DELAYED EXPOSURE AND,
   c. EFFECTS OF 30% VS. 80% SUBACUTE ENZYME INHIBITION

a. Dose Response Relationship for Acute Administration of Physostigmine

In this Second Annual Report, we present more detailed dose response curves for, supercontraction (Fig. 15) and the formation of "frothy" mitochondria after a single high dose injection of physostigmine (Fig. 16). Thirty minutes after a high dose of physostigmine (0.8-1.1 LD_{50}, 80-98% serum cholinesterase inhibition), <u>all</u> myofibers in the diaphragm and soleus muscles exhibited severe supercontraction in the subjunctional sarcoplasm, whereas few EDL myofibers exhibited supercontration. At 0.3 LD_{50} (80% inhibition), ultrastructural alterations are variable but consistent with <u>near or suprathreshold effect</u> (see for example Fig. 5). Evidence for toxic alteration differs for each cytoplasmic marker. Frothy or exploded mitochondria, swollen SR, Golgi, nuclear membranes occur at lower doses/earlier stages than does supercontraction. This is supported in part by the fact that in the absence of extrinsic stimulation, the Sol and EDL muscles were much less affected than the intrinsically stimulated diaphragm.

1) In the 1st Annual Report, we showed that at moderate, low, and very low doses (0.1, 0.01 and 0.001 LD_{50}, 67%, 33%, and 28% enzyme inhibition, respectively), little evidence for post-synaptic alterations was observed. None (0%) of the myofibers exhibited any evidence of supercontraction or for damage to mitochondria or sarcoplasmic reticulum (Figs. 16-18). At moderate doses (0.1 LD_{50}, 60-70% enzyme inhibition), <u>none</u> of the fibers examined in diaphragm, soleus, or EDL exhibited supercontraction. None exhibited "blistered" or "frothy" mitochondria or swollen sarcoplasmic reticulum in the motor endplate region. Thus, 0.1 LD_{50} (or 67% enzyme inhibition) appears to represent <u>subthreshold</u> for supercontraction/ mitochondrial damage in otherwise unstressed animals. Titration of responses revealed increasing ultrastructural alterations from 0.1 LD_{50} (67% $\pm$7% inhibition) to 0.3 LD_{50} (75% $\pm$5% inhibition) to 0.8 LD_{50} (89% $\pm$3% inhibition) (Figs. 15 and 16). Consequently, using a) supercontraction and b) "frothy mitochondria" as the <u>two morphological criteria</u> for establishing the desired "morphological dose response curve" for physostigmine toxicity, it is apparent that there is a steep onset occuring at "threshold" for the development of cytopathology. Thus, we propose that in resting, non-stressed animals, threshold for cytopathology occurs at 0.3 LD_{50} or 75 $\pm$5% enzyme inhibition. Whether threshold is static or is altered by muscle use (stimulation) is currently under investigation.

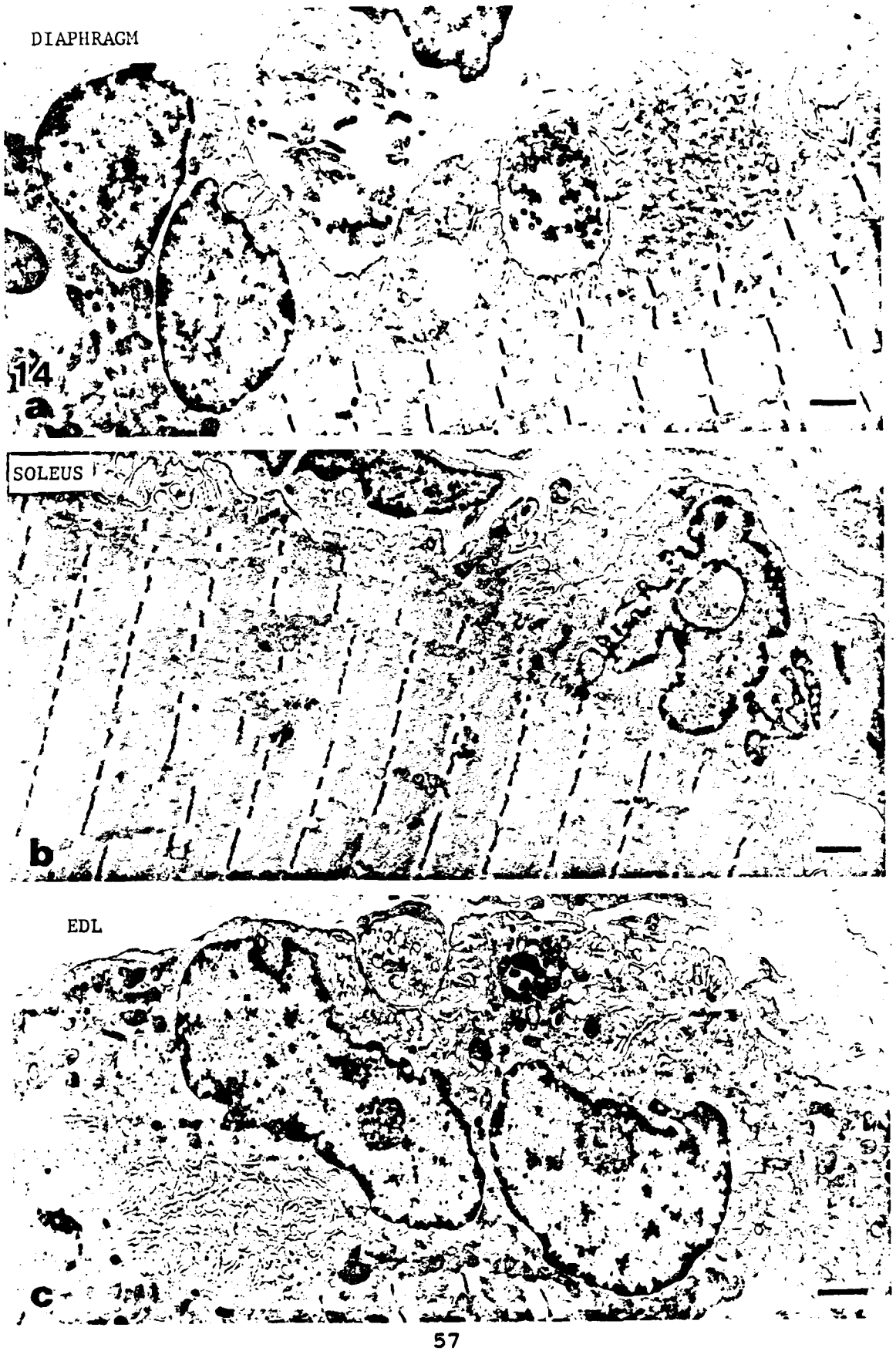
Figure 12. Effects of acute near threshold dose (0.3 LD_{50}) of physostigmine (80% serum ChE inhibition) on neuromuscular junctions of normally activated (not electrically stimulated) diaphragm (Fig. 12a) and 20 hz electically stimulated, soleus (Fig. 12b), and EDL muscles (Fig. 12c). About 60% of diaphragm neuromuscular junctions were supercontracted, and exhibited a continuum of severely to slightly damaged mitochondria (i.e., "exploded", "frothy", and "blistered"). Mitochondria also exhibited abnormal morphology in the nerve terminals. Sarcomeres of soleus and EDL appeared normal, but some mitochondria were "blistered" in the subjunctional cytoplasm.


DIAPHRAGM
12
a
SOLEUS
b
EDL
c

Figure 13. Effects of acute near threshold dose (0.3 LD_{50}) of physostigmine (80% serum ChE inhibition) on neuromuscular junctions of diaphragm (Fig. 13a), soleus (Fig. 13b), and EDL (Fig. 13c) under the stressful conditions produced by 40 hz electrical stimulation to EDL and soleus muscles only. 100% of diaphragm neuromuscular junctions were supercontracted and exhibited a continuum of severely to slightly damaged mitochondria. In contrast, sarcomeres of soleus and EDL appeared normal. However, in the subjunctional sarcoplasm of EDL severely damaged and/or frothy mitochondria, and swollen cisternae of cytoplasm membrane components were noted. Interestingly, soleus myofibers were less affected at 40 and 80 hz than at 20 hz (see Fig. 12).


DIAPHRAGM
13
a
SOLEUS
b
EDL
c

Figure 14. Effects of acute near threshold dose (0.3 LD_{50}) of physostigmine on neuromuscular junctions of diaphragm (Fig. 14a), soleus (Fig. 14b), and EDL (Fig. 14c) under the stressful conditions produced by 80 hz electrical stimulation to EDL and soleus muscles. In the diaphragm, frothy and blistered mitochondria were abundant in the subjuctional cytoplasm and into the intermyofibrillar space, but few exploded mitochondria were seen. This stage of morphological alteration may be indicative of impending supercontraction, even though myofibrils appeared normal. In the EDL muscle, myofibrils, mitochondria, Golgi complex, endoplasmic reticulum and outer nuclear membrane were swollen in the subjunctional cytoplasm. Abnormal mitochondria were also present in the nerve terminals of the EDL. This may reflect the increased rate and duration of EDL nerve terminal depolarizations by this non-tetanic stimulation vs reduced effect in soleus muscle occurring at well above tetanic fusion frequencies.


DIAPHRAGM
1/4
a
SOLEUS
b
EDL
c

15

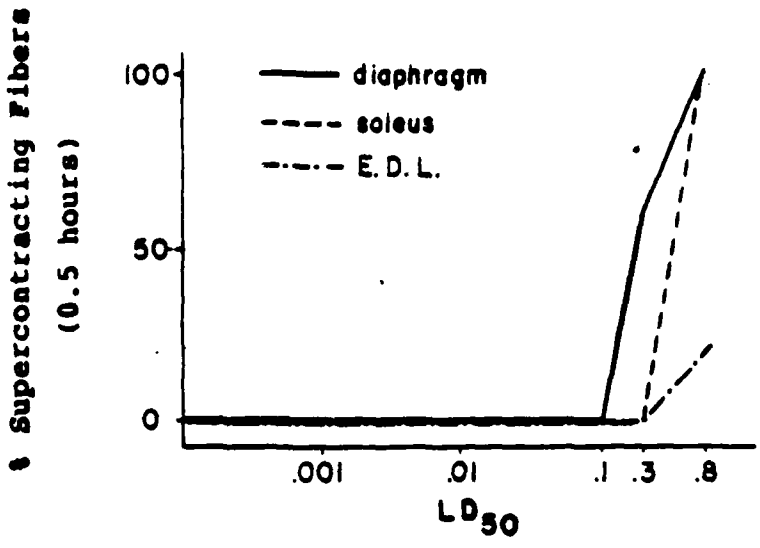


Figure 15. "Dose response curve" for percent of fibers with supercontraction 30 minutes PI at different levels of LD_{50} physostigmine. About 60% of fibers in the diaphragm muscles were supercontracted at a 0.3 LD_{50} dose of physostigmine, while soleus and EDL showed no supercontraction. At very high dose (0.8-1.1 LD_{50}) diaphragm and soleus muscles showed 100% supercontraction, whereas EDL muscle showed only 20% supercontraction.

16

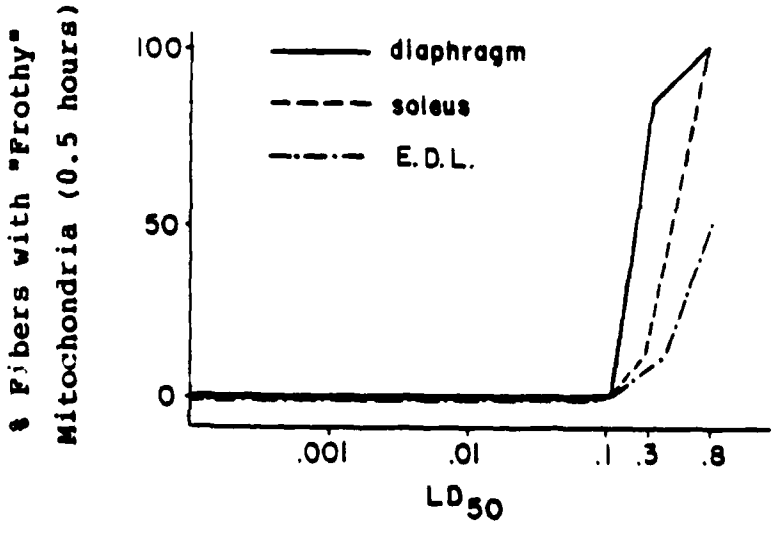


Figure 16. Dose response curves based on percent of "frothy" mitochondria induced by different dose levels of physostigmine. At 0.3 LD_{50} "frothy" mitochondria were found in 90% of subjunctional cytoplasm of diaphragm muscles, while only 10% of soleus and EDL had frothy mitochonria. At 0.8 LD_{50}, 100% of fibers in diaphragm and soleus had frothy mitochondria in the subjunctional cytoplasm, while about 50% of mitochondria were frothy in the EDL.

2) Moderate Dose (0.1 LD_{50})

After 30 minutes exposure to a moderate dose of physostigmine (0.1 LD_{50}, 67% inhibition), diaphragm myofibers revealed few gross changes in their myofibrils (Fig. 17). In most endplates, the nerve terminals appeared nearly "normal," with numerous synaptic vesicles, few coated vesicles, "normal" mitochondria (occasionally slightly swollen), and a few flattened cisternae, all indicative of normal or slightly increased presynaptic activity.

3) Low to Very Low Doses (0.01 and 0.001 LD_{50})

After 30 minutes exposure to low and very low doses of physostigmine (0.01 and 0.001 LD_{50}, 33% or less inhibition of blood ChE), myofibers from diaphragm, soleus, and EDL revealed no gross changes in nerve terminals or in the subjunctional cytoplasm (Figs. 18 and 19). Sarcomeres were of normal length and the membrane-bound organelles (mitochondria, sarcoplasmic reticulum, triads and T-system) appeared normal. The nerve terminals were filled with synaptic vesicles and occasional flattened cisternae. In most nerve terminals, mitochondria were normal, with only the "normal" number of small swellings. Thus, these data from animals treated with physostigmine are at variance with data from animals treated with pyridostigmine (Hudson, et al, 1983, who reported subtle alterations in endplate fine structure at ½hr PI in animals treated with 0.01 or 0.001 LD_{50}).

4) Threshold dose

At 0.3 LD_{50}, approximately 60% diaphragm myofibers were supercontracted, while soleus and EDL myofibers showed no evidence for supercontraction (See Fig. 15). (This additional exposure level was not required for completion of our contract, but was selected to provide essential details concerning drug toxicity at or near the "threshold" level.)

Figure 17. Effects of an acute moderate dose of physostigmine (0.1 LD_{50}, 67% serum ChE inhibition) on neuromuscular juncitons 30 minutes PI. Myofibers of diaphragm (Fig. 17a) and EDL (Fig. 17c) muscles exhibited virtually normal ultrastructure. However, the subjunctional sarcomeres of soleus muscle (Fig. 17b) showed slightly irregular or "zig-zag" Z bands. At this dose, most mitochondria appear normal in both subjunctional sarcoplasm and nerve terminals.

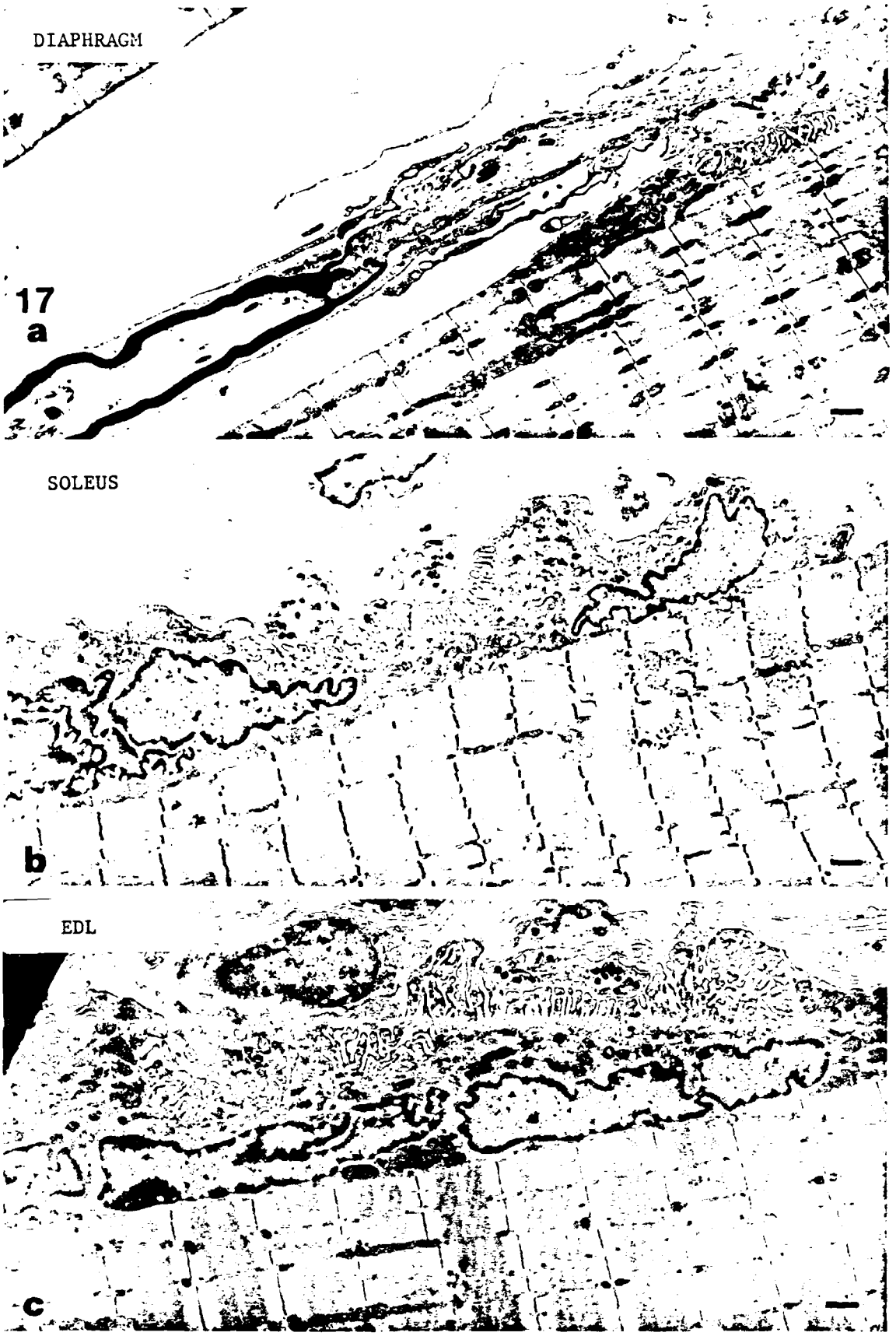

DIAPHRAGM
17
a
SOLEUS
b
EDL
c

Figure 18. Effects of an acute low dose of physostigmine (0.01 LD_{50}, 33% inhibiton) on neuromuscular junctions 30 minutes PI. Myofibers and nerve terminals in the neuromuscular *junctionsl of diaphragm* (Fig. 18a), soleus (Fig. 18b), and EDL (Fig. 18c) exhibited apparently normal ultrastructure. (The few swollen mitochondria in some nerve terminals are believed to reflect preparation artifacts.)

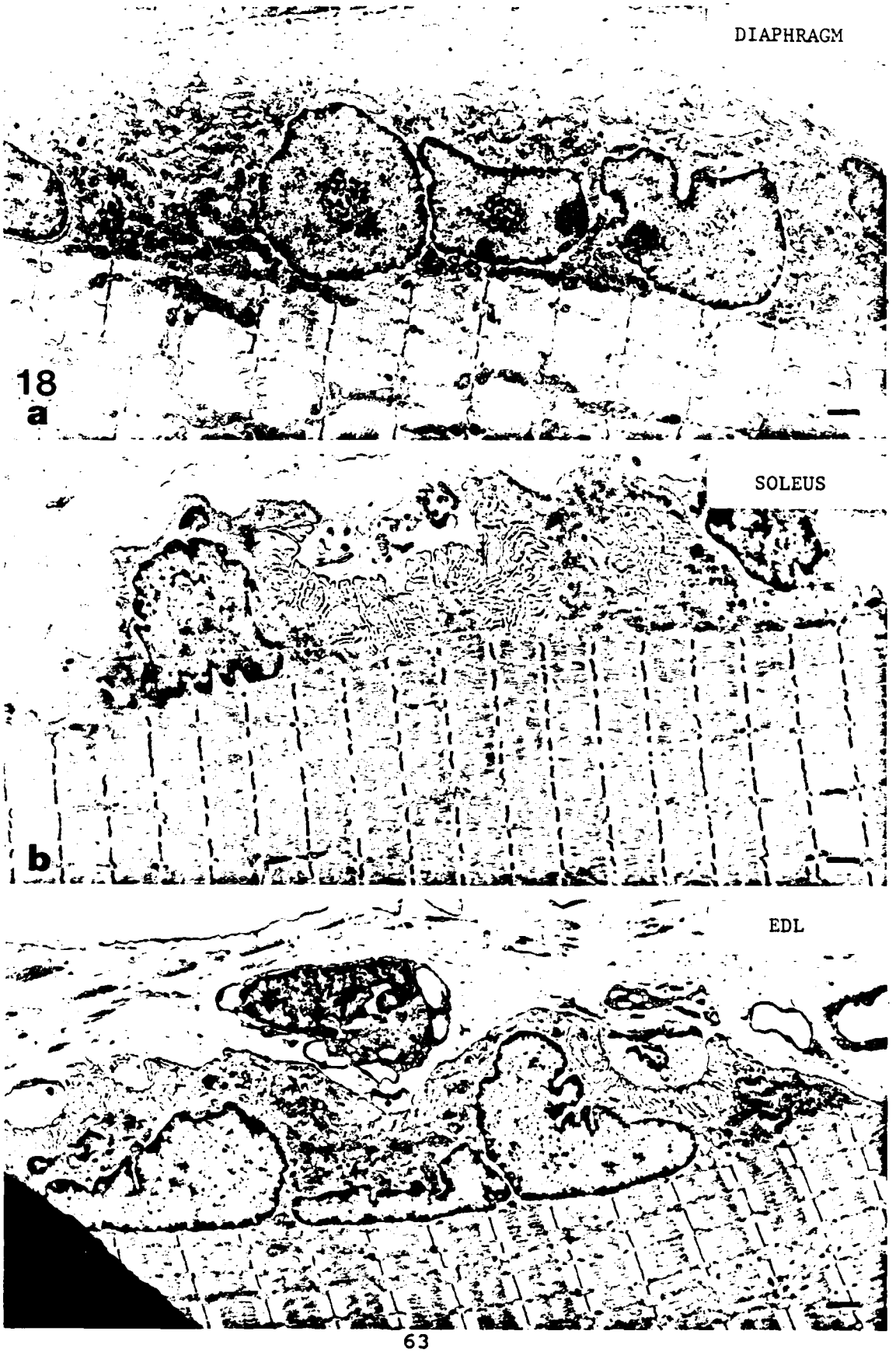
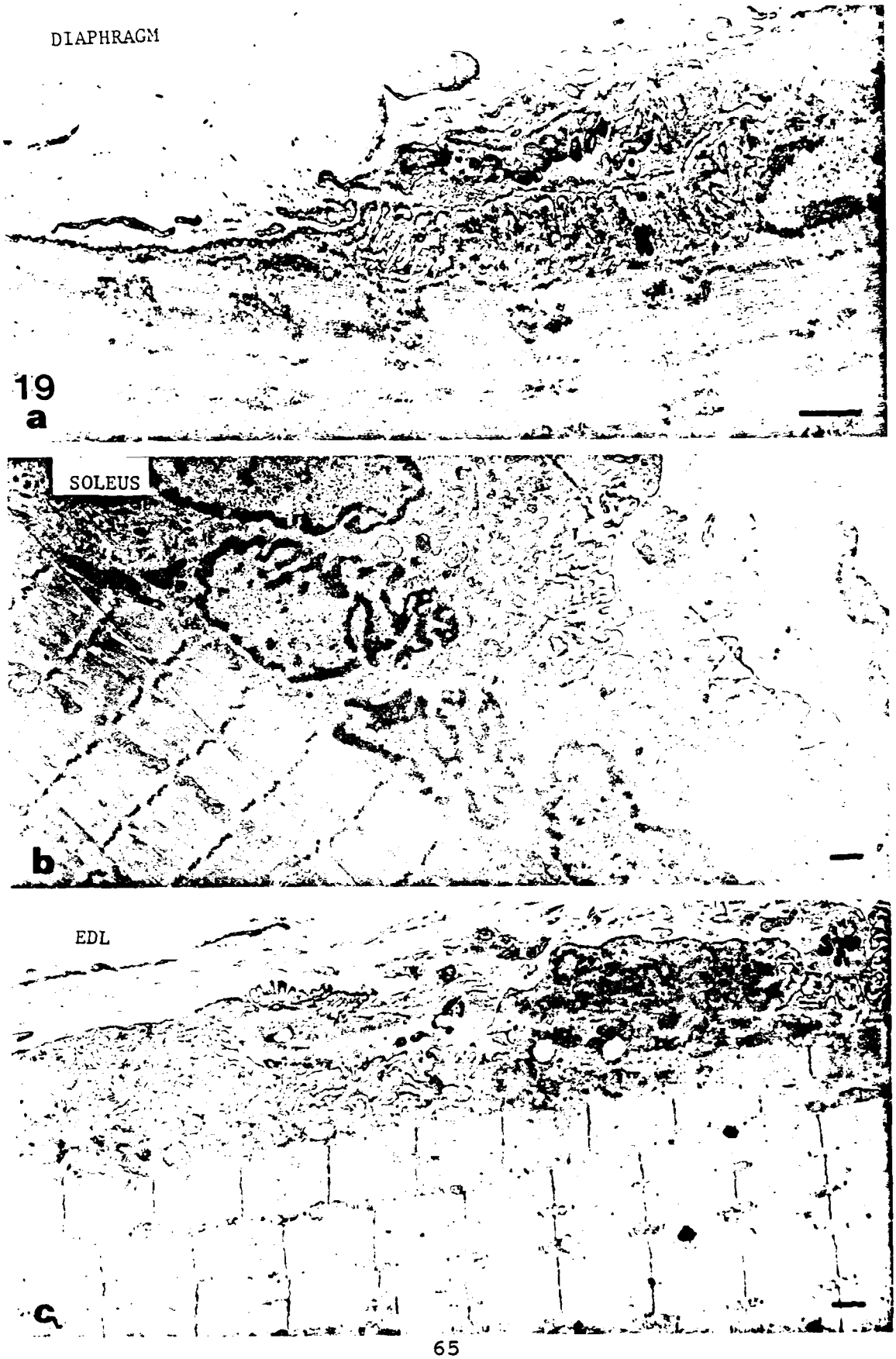

DIAPHRAGM
18
a
SOLEUS
b
EDL

Figure 19. Effects of an acute very low dose of physostigmine (0.001 LD_{50}) on neuromuscular junctions 30 minutes PI. Fast twitch myofibers of diaphragm (Fig. 19a) and EDL (Fig. 19c) appeared normal but myofibers of the slow twitch soleus muscle (Fig. 19b) exhibited slightly irregular Z bands (this may be normal). A few distended mitochondria were seen in the sujucntional cytoplasm and nerve terminals of some EDL myofibers. Again, this particular type of swollen mitochondria is attributed to artifact of hypoxia and/or gluteraldehyde fixation. (See ref. 35 and 57)


DIAPHRAGM
19
a
SOLEUS
b
EDL
c

b. DOSE-RESPONSE STUDIES OF ACUTE-DELAYED EFFECTS

1) High Dose

In the first Annual Report, we showed that after a single high dose injection (0.8-1.1 LD_{50}), several types of damage/alteration persist or continue to accumulate during the period 1-56 days PI. (In many instances, it is not possible to differentiate between changes representing acute-delayed effects [TASK I] and changes representing recovery/repair. Therefore, please refer also to Section 7, TASK IV, below.) As described in section 3.a.1, above, destructive effects on sarcomeres and subjunctional mitochondria were consistently observed in the neuromuscular junctions of diaphragm and soleus muscles 30 minutes after a single 0.8-1.1 LD_{50} injection of physostigmine, but were minimal in EDL (see Fig. 6). These effects were essentially reversed in diaphragm and soleus muscle by 24 hours PI (Figs. 20a and 20b, and see also TASK IV, Reversibility). In the EDL, however, the most severe damage occurred between 30 minutes and 24 hours post injection (Fig. 20c). That is the period when the rats were recovering and had begun to use the EDL muscles for walking. Thus, we concluded that the delayed effects of physostigmine on EDL may be related to the resumption of voluntary muscle activity at 1-6 hrs PI rather than to fiber-type differences in drug susceptibility. At 7 days and beyond (Figs. 21-24), repair processes/delayed effects were essentially identical in the three muscles. However, at 14, 28 and 56 days PI, (Fig.s 25-27), approximately 30% of myofibers in diaphragm showed evidence for partial denervation, including the formation of vesicular debris in the clefts (Fig.s 25 and 26) and the disappearance/retraction of nerve terminal branches (Figs. 25 and 27). For example, vesicular debris was occassionally found above residual junctional folds that were devoid of attached nerve terminals (Figs. 25b and 25c), similar to images obtained from patients with myasthemia gravis. For additional details, see 1st Annual Report.

2) Delayed Effects From Moderate to Very Low Doses of Physostigmine

At 24 hours PI, no damage to endplates was detected and no acute-delayed effects were noted at 0.1, 0.01, or 0.001 LD_{50} (Figs. 28-30). Moreover, from 1 to 56 days PI no ultrastructural or physiological changes in muscle were detected in any of several hundred myofibers examined. (Fig. 28 for 0.1 LD_{50}; Fig. 29 for 0.001 LD_{50}; and Fig. 30 for 0.0001 LD_{50}). In all well fixed muscle samples, subjunctional sarcomeres exhibited the same length as extrajunctional sarcomeres; pre- and post-synaptic mitchondria and other membrane bound organelles appeared normal; junctional fold depth, number and distribution appeared normal; and Schwann cells had the same variability as in untreated controls. Similarly, delayed ultrastructural alterations were not observed at any stage from 1-56 days PI. (Also, see next section 7a, "Reversibility". In that section, also we show that there is no detectable evidence for delayed neuromuscular alterations at 1-56 days following a single moderate to low dose injection [i.e., no "acute-delayed" effects following 0.1 LD_{50} to 0.01 LD_{50}]. Micrographs and graphs in that section illustrate "recovery" from 1-56 days PI.)

Figure 20. Effect of an acute high dose of physostigmine (0.8-1.1 LD_{50}) on diaphragm, soleus, and EDL neuromuscular junctions 1 day PI. In the diaphragm muscle (Fig. 20a), the only effect remaining from the severe supercontraction noted ½hr PI (see in Fig. 4 and 6a) was an irregular arrangement of myofibrils seen just below the junctional folds. The myofibrils appeared almost fully recovered from supercontraction. There were no swollen or frothy mitochondria remaining. In the soleus muscle (Fig. 20b), the subjunctional myofibrils also appeared normal. However, in the EDL (Fig. 20c), evidence of increased damage to myofibrils was detected. Very irregular Z bands and misaligned myofilaments, as well as the presence of frothy mitochondria, indicated that additional damage occurred in EDL fibers between 30 minutes and 24 hours. We infer that in the EDL, severe damage occurred after ½hr and within 6 hours of injection (i.e., before inactivation of physostigmine, ref. 1), but that much of the damage had been repaired by 1 day PI.

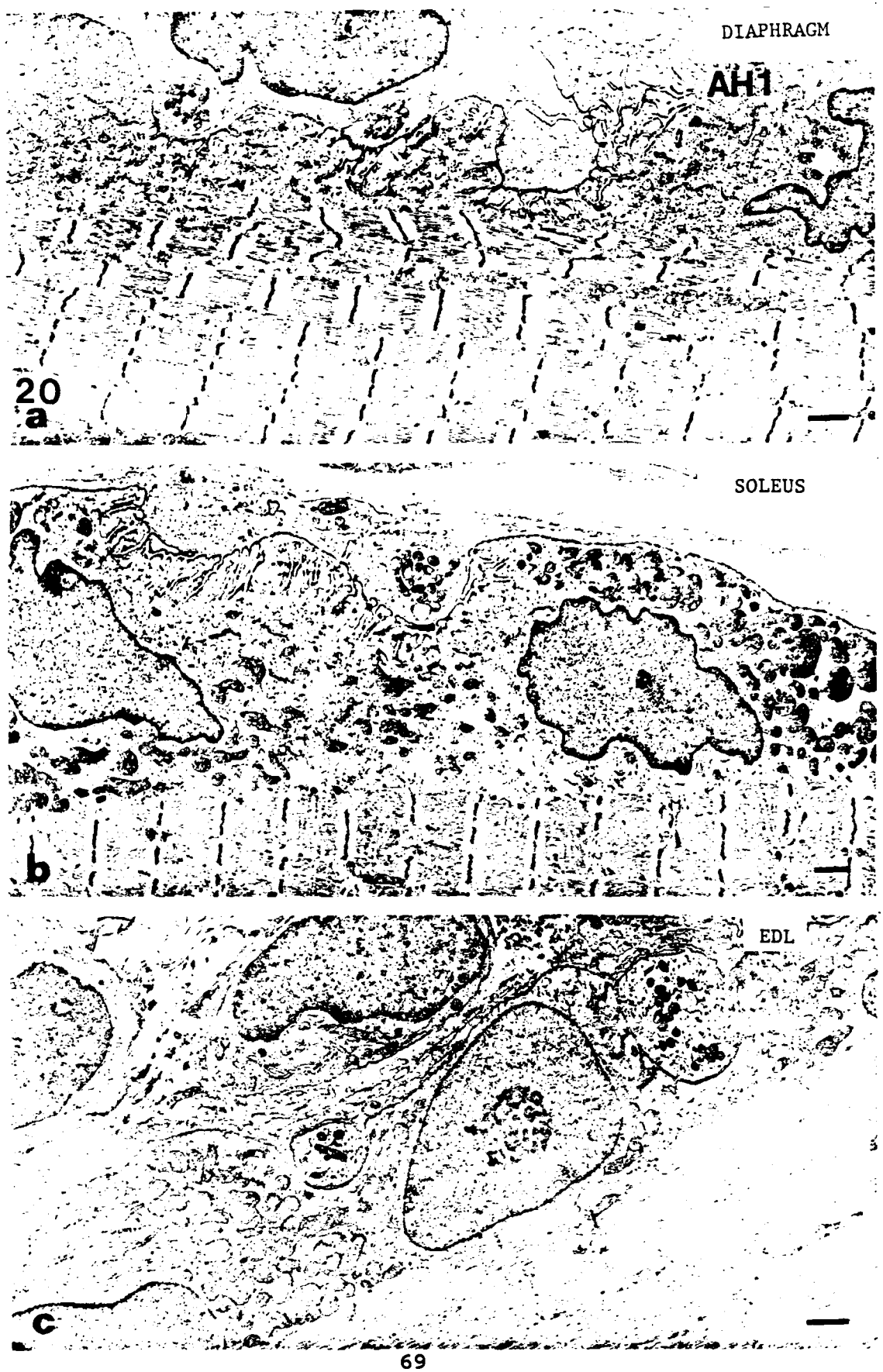

DIAPHRAGM
AH1
20
a
SOLEUS
b
EDL
c

Figure 21. Recovery of neuromuscular junctions in diaphragm (Fig. 21a), soleus (Fig. 21b), and EDL (Fig. 21c) 7 days after acute high-dose physostigmine (0.8-1.1 LD_{50}) exposure. All myofibrils, mitochondria, and nerve terminals appeared normal. (The swollen mitochondria in Fig. 21b are typical fixation artifacts).


DIAPHRAGM
AH-R7
2.1
a
SOLEUS
b
EDL
c

Figure 22. Recovery of neuromuscular junctions in diaphragm (Fig. 22a), soleus (Fig. 22b), and EDL (Fig. 22c) 14 days after acute high-dose physostigmine (0.8-1.1 LD_{50}) exposure. All myofibrils, mitochondria, and nerve terminals appeared normal.

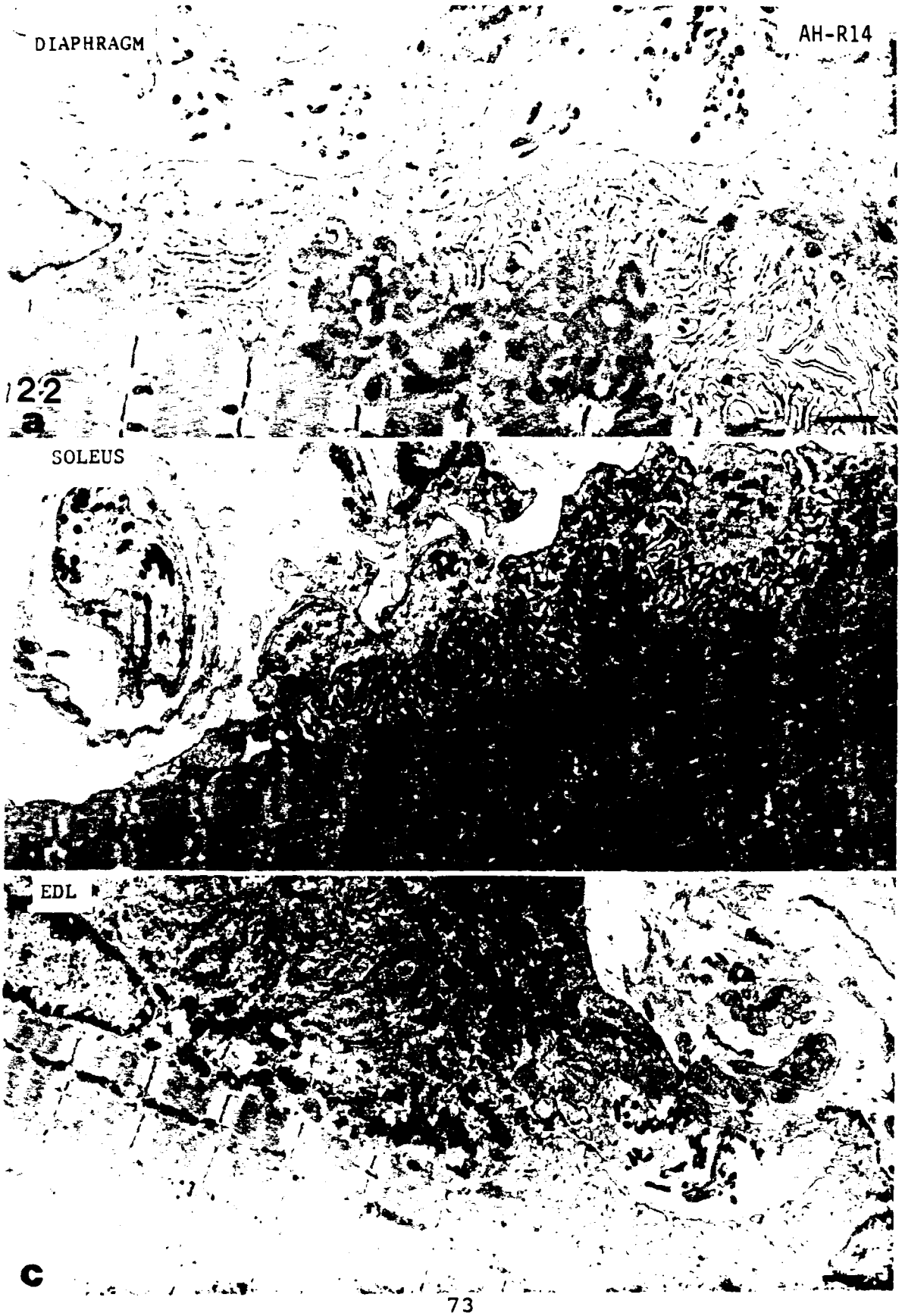

DIAPHRAGM
AH-R14
22
a
SOLEUS
EDL
c

Figure 23. Recovery of neuromuscular junctions in diaphragm (Fig. 23a), soleus (Fig. 23b), and EDL (Fig. 23c) 28 days after acute high-dose physostigmine (0.8-1.1 LD_{50}) exposure. Myofibrils, mitochondria, and nerve terminals were normal. (The swollen mitochondria in Fig. 23b and c are typical fixation artifacts). The arrow in Figure 23a points to the necrotic residue of a nucleus, indicating that at least some nuclei were irreversibly damaged by the initial prolonged and severe endplate depolarizations. The arrowheads in Figure 23a indicate vesicular debris similar to that reported in neuromuscular junctions from human patients with myasthenia gravis.

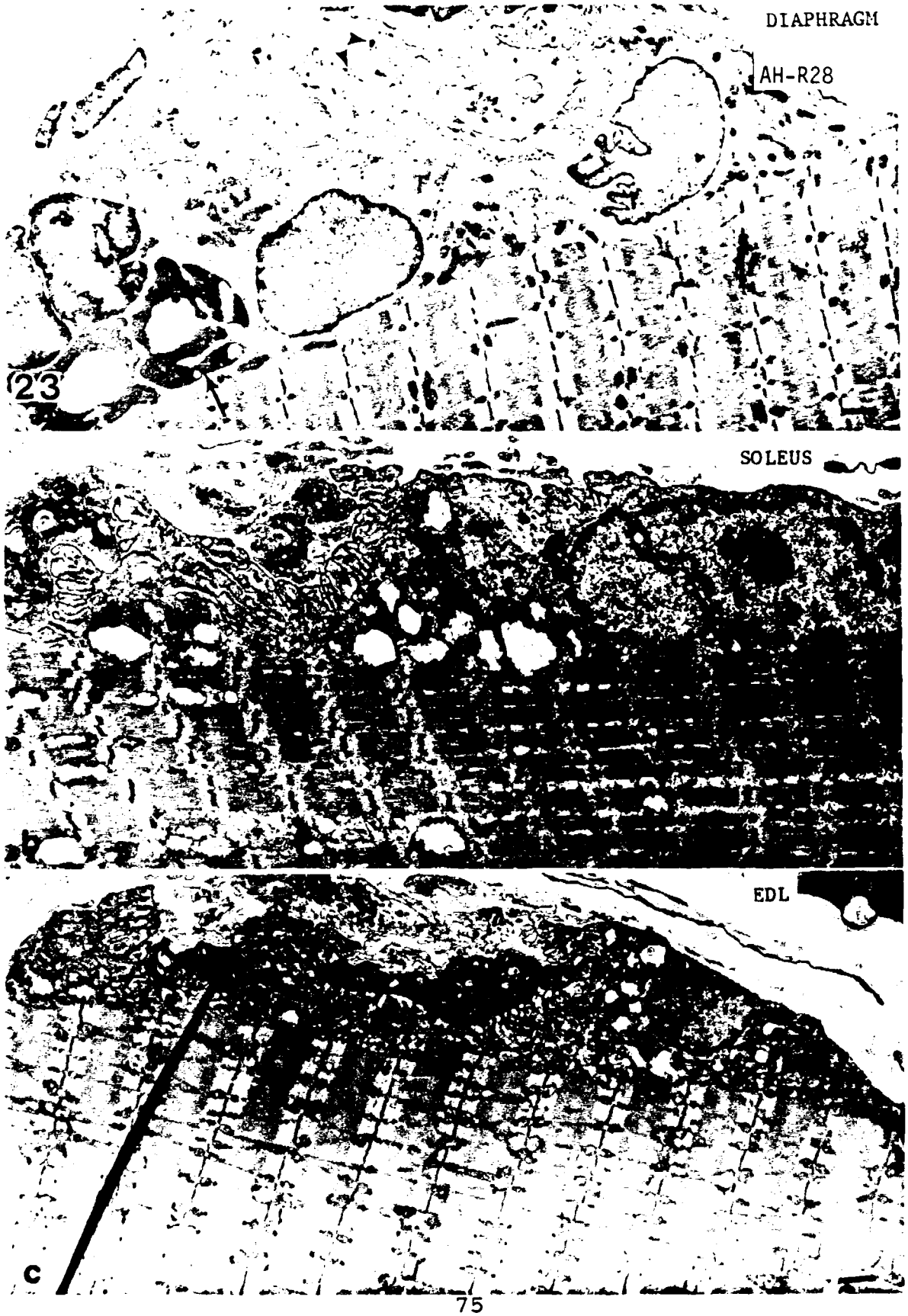

DIAPHRAGM
AH-R28
23
SOLEUS
EDL
C

Figure 24. Recovery of neuromuscular junctions in diaphragm (Fig. 24a), soleus (Fig. 24b), and EDL (Fig. 24c) 56 days after acute high-dose physostigmine (0.8-1.1 LD_{50}) exposure. No detectable alterations were found in the myofibrils, mitochondria, or nerve terminals.

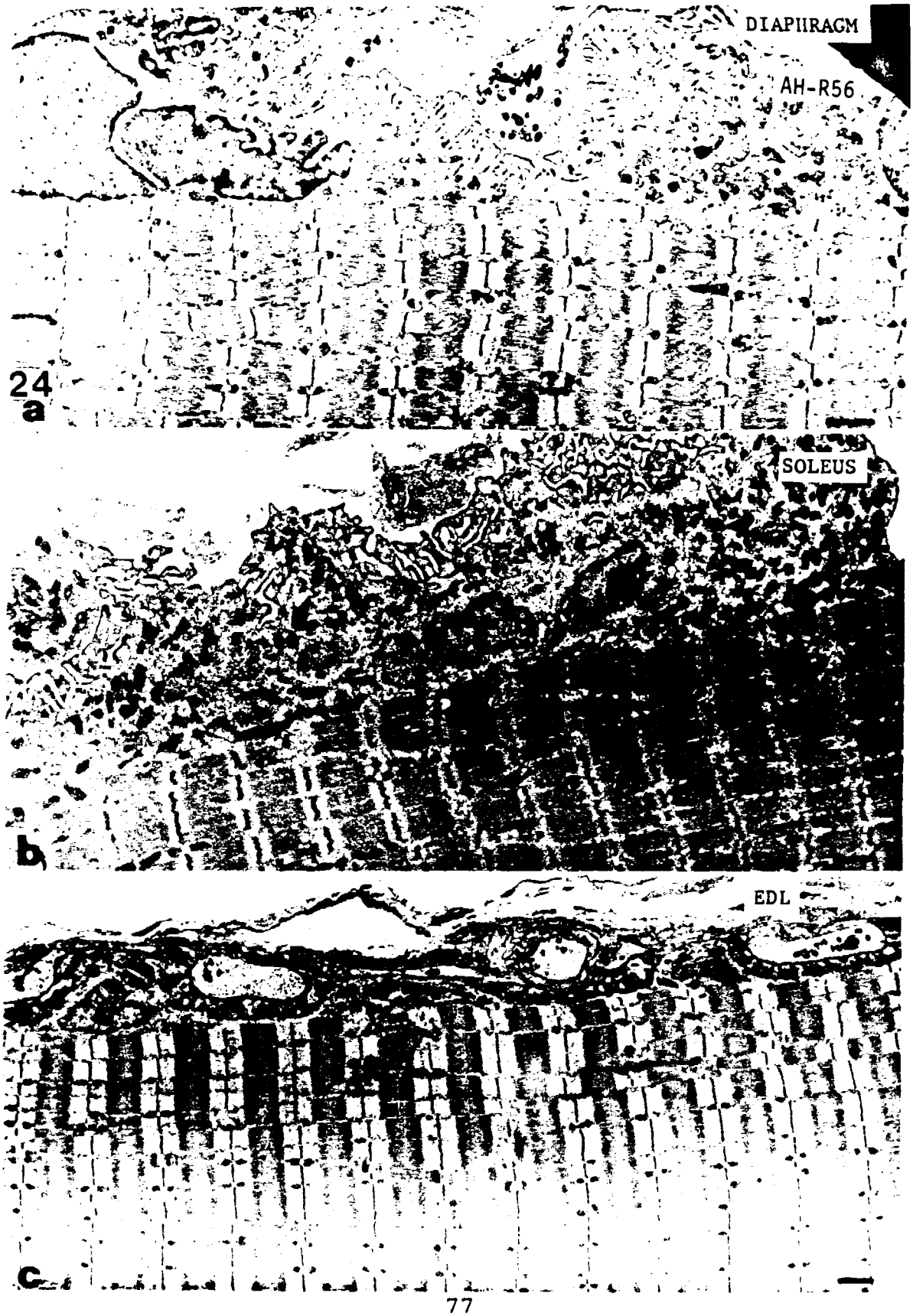

DIAPHRAGM
AH-R56
24
a
SOLEUS
b
EDL
c

Figure 25. Delayed effects on the nerve terminals of diaphragm muscle after a single acute dose injection of physostigmine. Micrographs show neuromuscular junctions of diaphragm muscles 14 (Fig. 25a), 28 (Fig. 25b), and 56 days (Fig. 25c) PI. Debris (arrowheads) similar to that reported in a neuromusclular junction from human patients with myasthenia gravis and of rats treated with anti-ChE agents (see text) are found in the synaptic cleft (Fig. 25a), on the denervated surface of the junction (Fig. 25b), and on the denervated surface of a degenerated endplate (Fig. 25c).

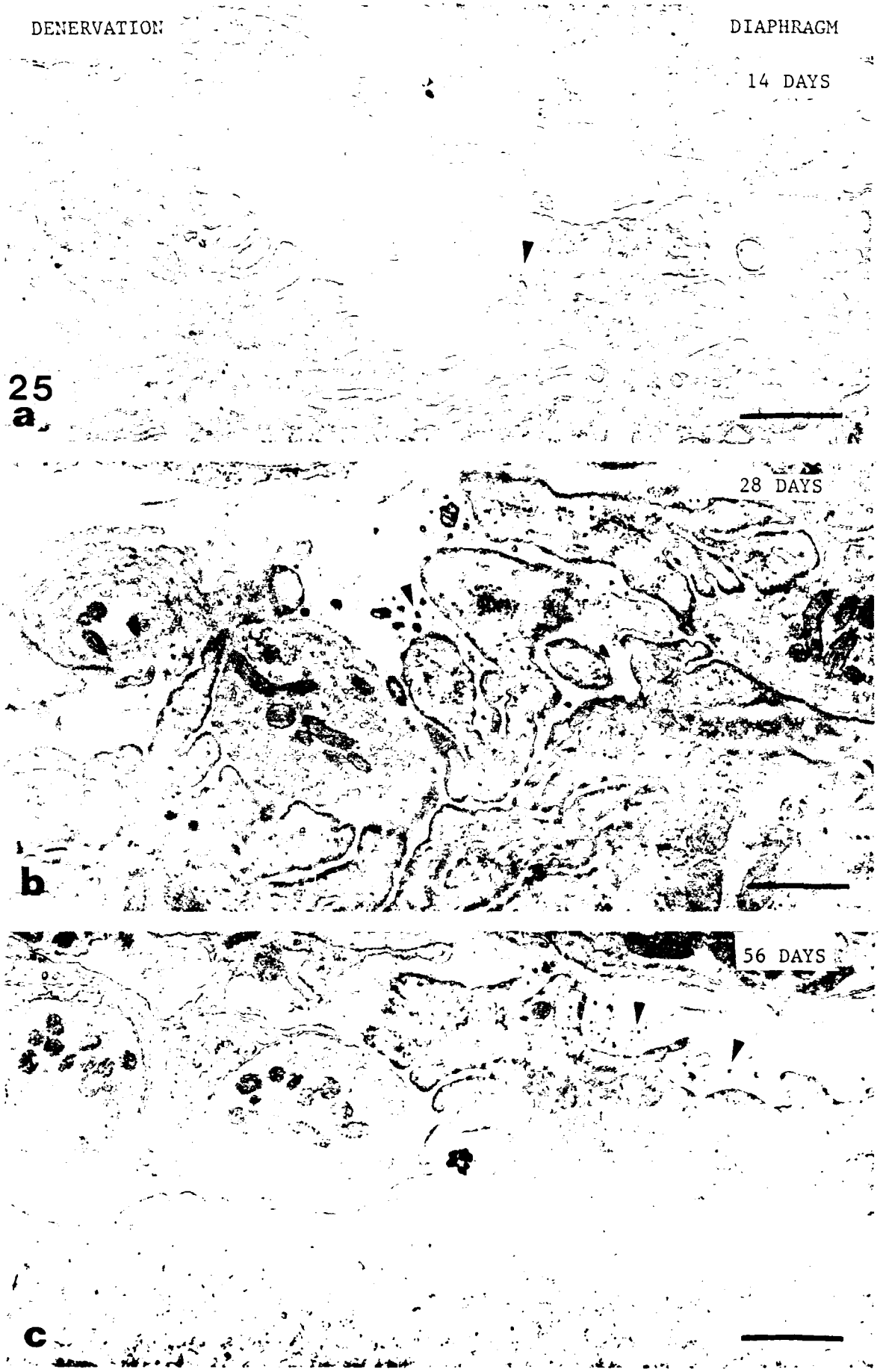
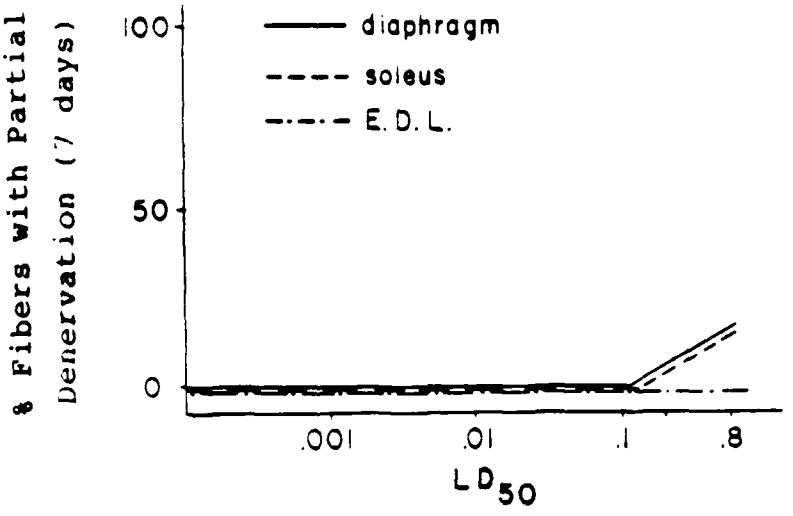

DENERVATION
DIAPHRAGM
14 DAYS
25
a
28 DAYS
b
56 DAYS
c

Figure 26. "Dose response curves" of endplates with areas of junctional fold destruction and resulting formation of vesicular debris.

Figure 27. "Dose response curves" for acute delayed effects of physostigmine. At 7 days PI, none (0%) of the fibers exposed to very low to moderate doses showed any evidence of denervation. However, 7 days after 0.8 LD_{50}, images from approximately 20% of diaphragm and soleus myofibers showed evidence of denervation (see text). Since denervated folds occupy only a small portion of affected endplates, it could be expected that serial sectioning techniques would reveal a substantially higher percent of denervated areas in all muscles exposed to 0.8 LD_{50}.

26


% Fibers with Partial Denervation (7 days)
100
50
0
diaphragm
soleus
E.D.L.
.001
.01
.1
.8
LD50

27

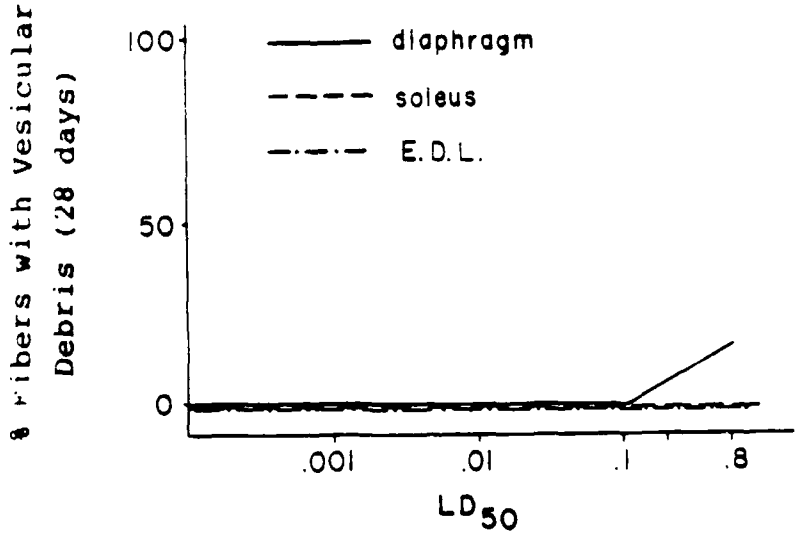

% Fibers with Vesicular Debris (28 days)
100
50
0
diaphragm
soleus
E.D.L.
.001
.01
.1
.8
LD50

Figure 28. Acute delayed effects of low-dose physostigmine exposure (0.1 LD_{50}) on the neuromuscular junctions of diaphragm (Fig. 28a), soleus (Fig. 28b), and EDL (Fig. 28c) 1 day PI. Myofibrils, mitochondria, and nerve terminals appear normal. Although diaphragm and soleus myofibers had been slightly affected at 30 minutes PI (Fig. 17a,b), it is clear that these fibers have virtually recovered from any minor alterations at ½hr.

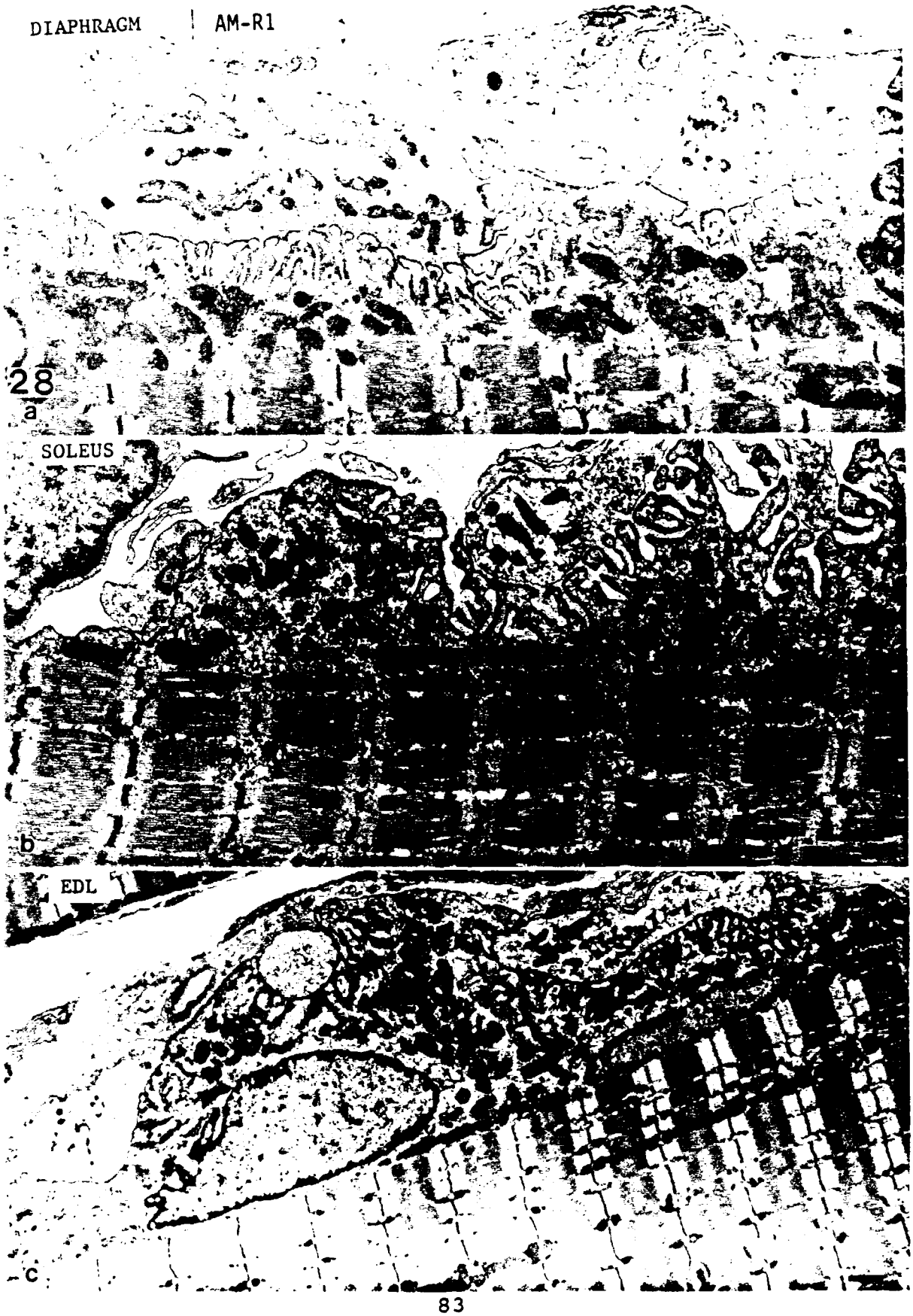

DIAPHRAGM
AM-R1
28
a
SOLEUS
b
EDL
c

Figure 29. Acute delayed effects of physostigmine (0.01 LD_{50}) on the neuromuscular junctions of diaphragm (Fig. 29a), soleus (Fig. 29b), and EDL (Fig. 29c) 1 day PI. Myofibrils, mitochondria, and nerve terminals appear normal. Swollen mitochondria in some nerve terminals are probably fixation artifacts.


DIAPHRAGM
AL-R1
29
SOLEUS
b
EDL

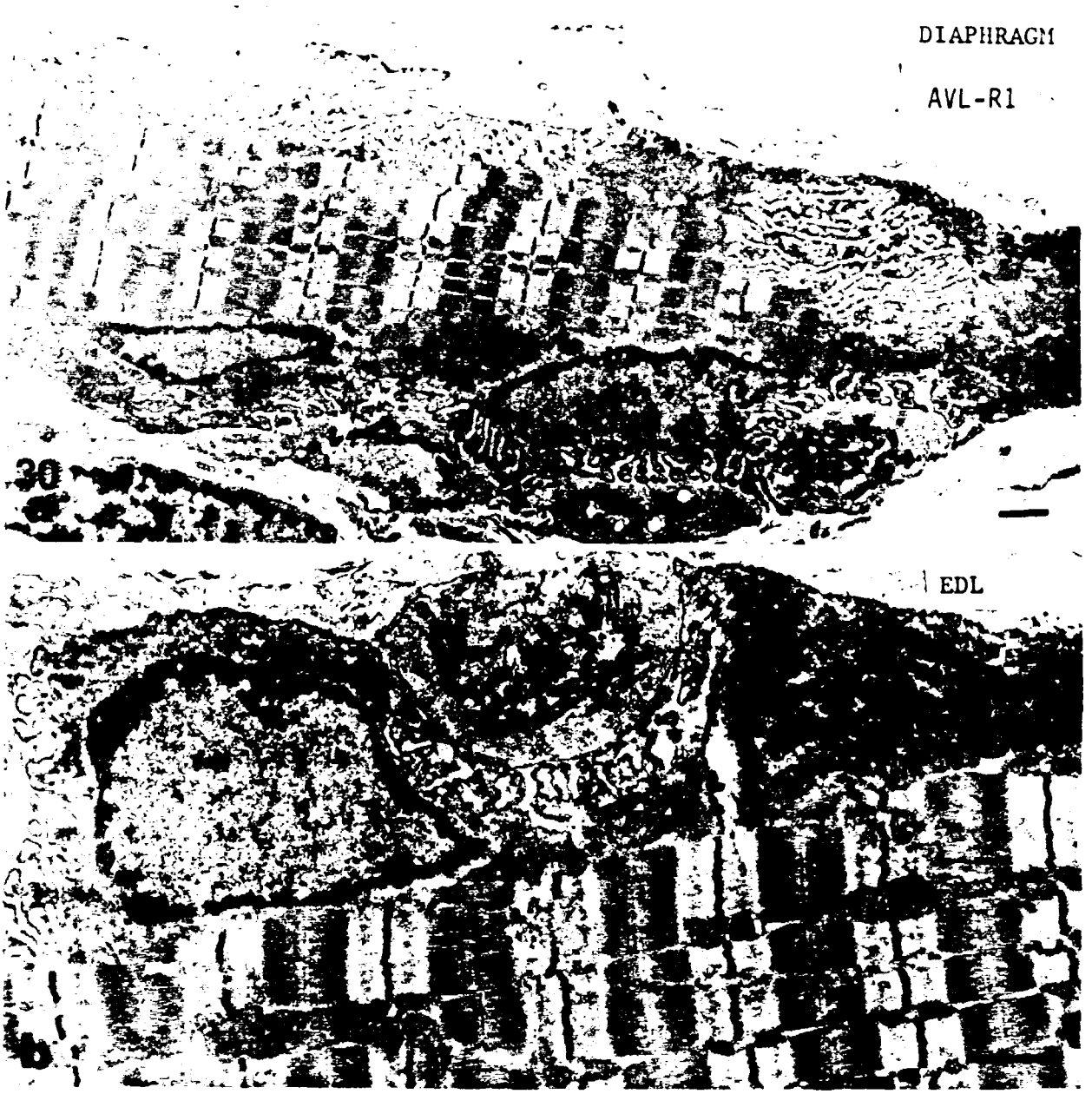


Figure 30. Acute delayed effects of physostigmine (0.001 LD_{50}) on the neuromuscular junctions of diaphragm (Fig. 30a) and EDL (Fig. 30b) 1 day PI. Myofibrils, mitochondria, and nerve terminals appear normal. Thus, no signs of delayed toxicity were observed in the muscles of animals treated with low doses.

c. Dose Response Effects of Subacute Exposure

Sufficient data are now available to permit initial qualitative and quantitative assessment of this portion of TASK III. No alterations were observed at any time (1-14 days) during subacute exposure to a low dose of physostigmine (30-50% serum ChE inhibition). Surprisingly, very few fibers in the high dose group (80% inhibition for up to 14 days as well as for up to 28 days of recovery from high dose subacute exposure) exhibited any signs of physostigmine induced neuromuscular pathology. Although some soleus fibers had evident myofiber damage at 7 days, none exhibited supercontraction. These data are shown graphically as "dose response curves" (Fig. 31) and as a graph of temporal changes in ultrastructure following subacute exposure (Fig. 32)

7. **TASK IV:** REVERSIBILITY OF ULTRASTRUCTURAL ALTERATIONS

a. Following Acute Exposure

1) Reversibility of Acute High Dose Effects

As indicated in Section 6.b. (pp 66-80) above, from 24 hours to 14 days after a single high dose injection (0.8-1.1 LD_{50}), diaphragm and soleus fibers exhibited decreasing residual effects of the initial supercontractions (Figs. 20-27). At 24 hours PI (Fig. 20), neuromuscular junctions of diaphragm and soleus muscles were well recovered from drug effect, no supercontraction was detected, and myofibrils had well ordered cross-striations. However, some subjunctional sarcomeres remained somewhat irregular and misaligned in some fibers. In contrast, at 24 hours post injection, EDL myofibers exhibited increased evidence for supercontraction (Fig. 20c). However, in all fibers the acute effects of 0.8-1.1 LD_{50} were substantially reversed by 7 days (Fig. 21) and virtually indetectable by 14 days and thereafter (Figs. 22-25). An additional form of "reversibility" from acute high dose exposure was observed as "reinnervation". In less than 25% of diaphragm and soleus neuromuscular junctions, very small brances of nerve terminal were observed, often not embedded in a recognizable primary synaptic groove (Fig. 33 and 34). As suggested in the First Annual Report, such images are similar to those found during denervation and secondary reinnervation by "collateral sprouting".

To create time courses of "Recovery" curves for "Reversibility of acute-delayed" effects, we have graphed the relative number of fibers with "normal myofibers" (i.e., those without evidence for supercontraction) as criteria for establishing morphological "dose response curves" following both high dose (Fig. 35a) and moderate to very low dose (Fig. 35b. Each data point is based on approximately 8-10 fibers analyzed.) Micrograph records for moderate to very low dose exposures are shown in the next section as Figures 36-43.

It is clear from these data that recovery from damage to myofibers is rapid. Note: However, that in the First Annual Report, we also showed that other secondary effects persist or accumulate in a few fibers for up to 56 days.

31

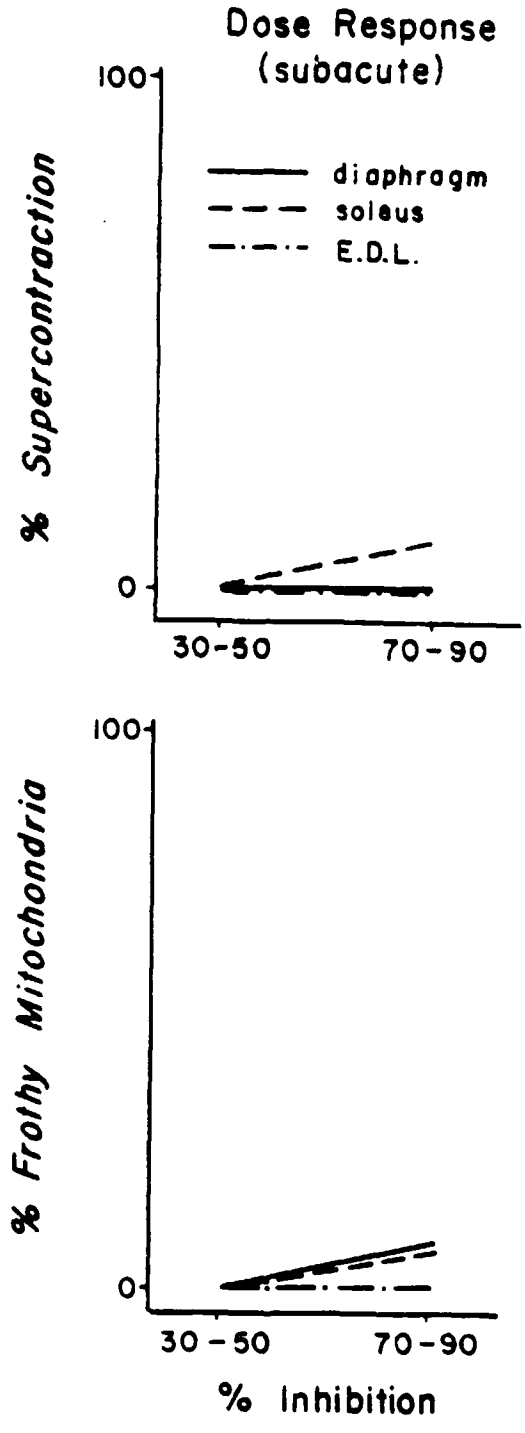


Figure 31. Dose response curves for low and high dose subacute exposures to physostigmine. At low dose, no changes were observed in sarcomere or mitochondrial ultrastructure. At high dose, approximately 10% of sarcomeres were at least partially disrupted and most of those same fibers had damaged mitochondria.

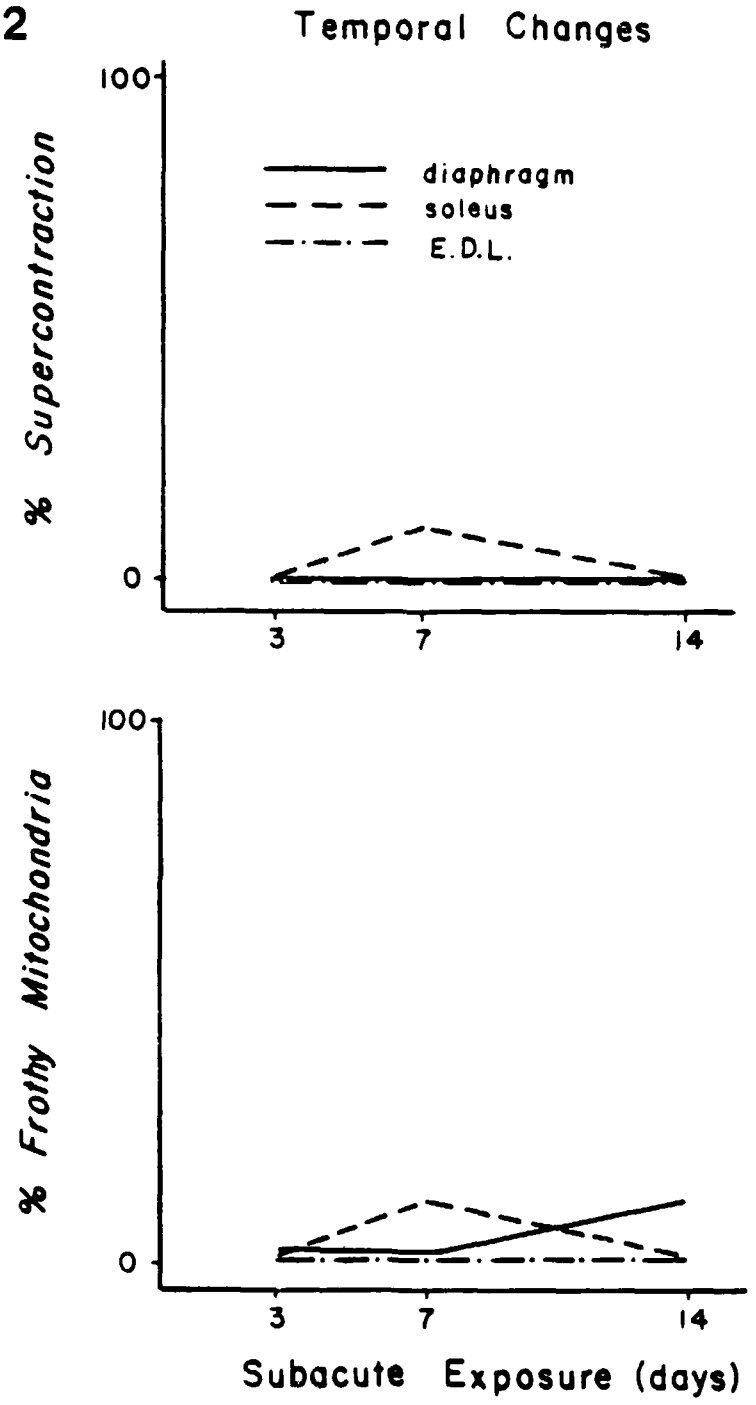
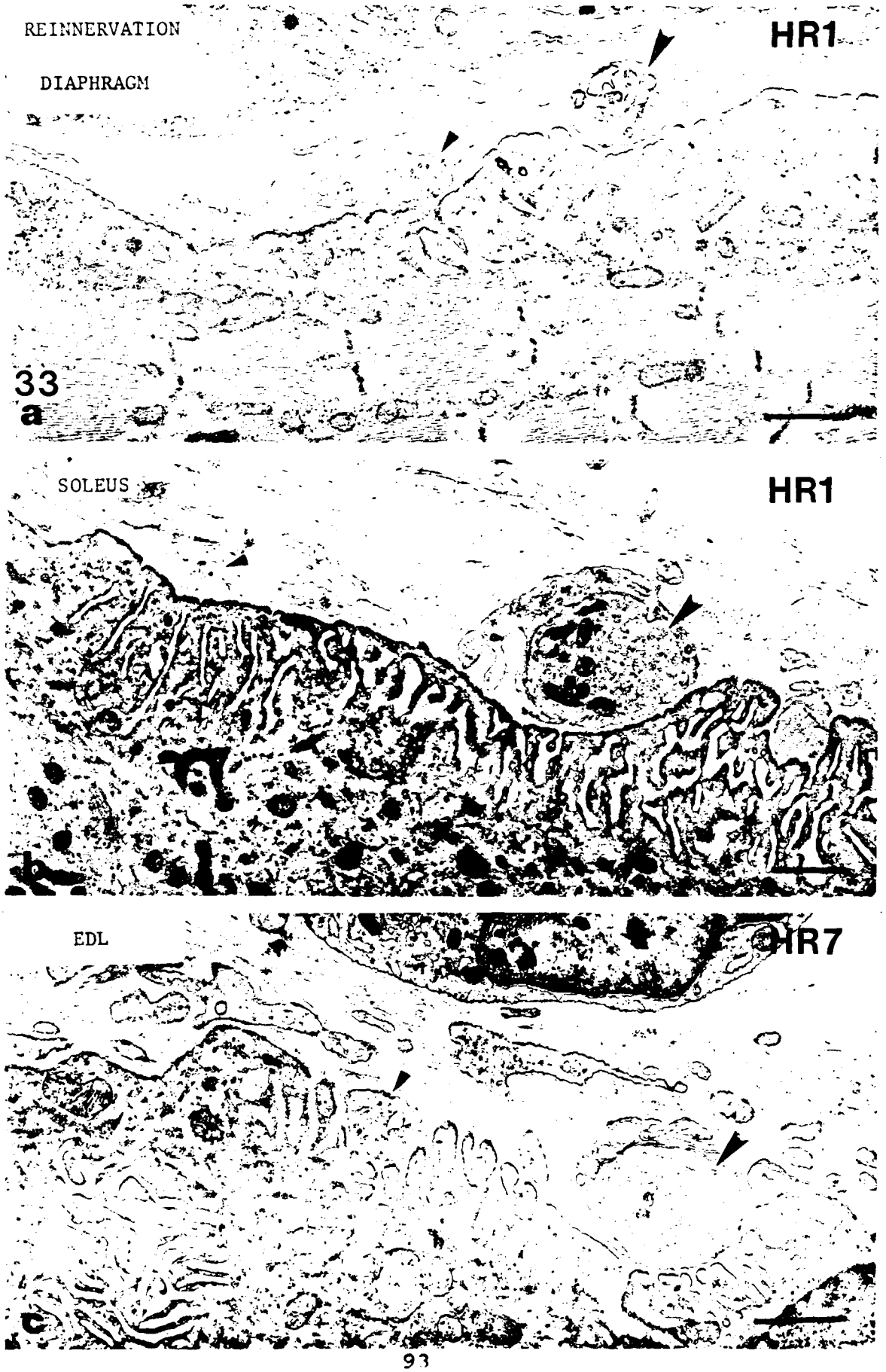


Figure 32. Myofiber changes occurring after 3, ,7 and 14 days subacute exposure to a continuaous infusion of a high dose of physostigmine (7 LD_{50}/day or 0.3 LD50/hr). Surprisingly few myofibers showed evidence for cytopathology.

Figure 33. Delayed effects of acute high dose exposure to physostigmine (0.8-1.1 LD_{50}) on the nerve terminals of diaphragm (Fig. 33a), and soleus (Fig. 33b) muscles 1 day PI and of EDL muscles (Fig. 33c) 7 day PI. Small nerve terminals (small arrowheads) and larger nerve terminals (large arrowheads) were observed on and adjacent to flattened denervated portions of the junction. These micrographs are consistent with processes of denervation and reinnervation of nerve terminals by collateral "sprouting".


REINNERVATION
DIAPHRAGM
HR1
33
a
SOLEUS
HR1
EDL
HR7
c

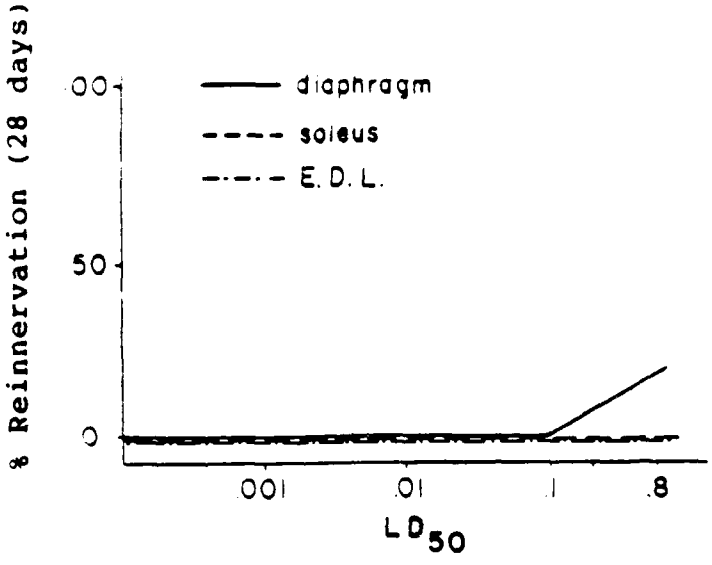
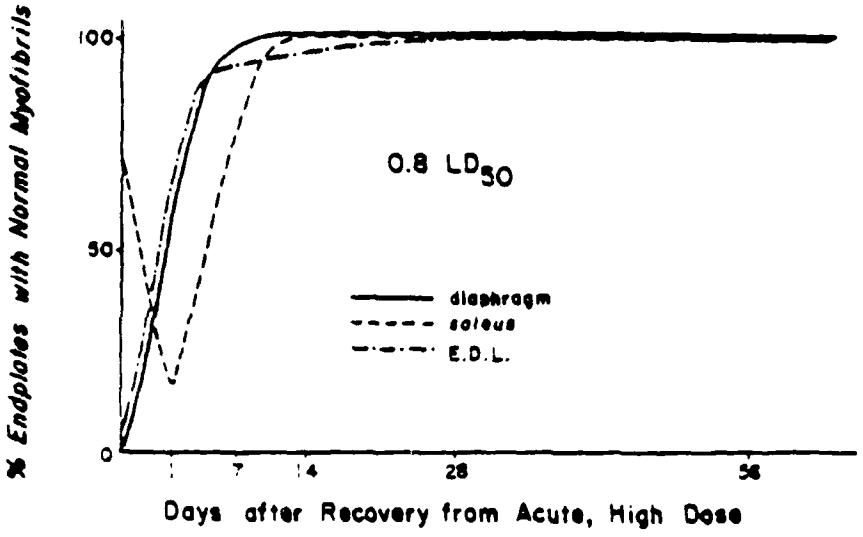
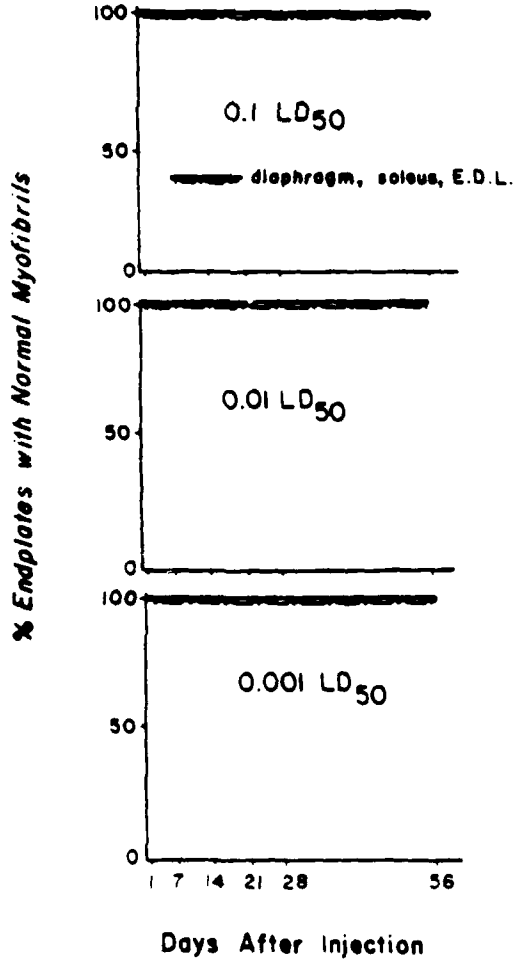


Figure 34. Relative frequency of endplates with small diameter nerve processes not embedded in a primary synaptic cleft. Because of the limited area involved in "collateral sprouting" and/or reinnervation, these probably represent lower limit estimates.

Figure35a. Temporal response curves relative number (%) of fibers with normal subjunctional myofibers in diaphragm, soleus, and EDL muscles after high dose (8-1.1 LD_{50}) injections of physostigmine. One hundred percent of diaphragm and soleus myofibers show supercontraction at 30 minutes PI. By 7 days, virtually all have recovered. In contrast, EDL myofibers show more damage at 1 day PI and slightly slower rate of recovery, but with virtually complete recovery by 14 days PI.

Figure 35b-d. At lower doses of physostigmine (0.1-00.1 LD_{50}), all diaphragm, soleus and EDL myofibers were normal at all stages of "recovery".


% Endplates with Normal Myofibrils
100
50
0
0.8 LD50
diaphragm
soleus
E.D.L.
1
7
14
28
56
Days after Recovery from Acute, High Dose


% Endplates with Normal Myofibrils
100
50
0
0.1 LD50
diaphragm, soleus, E.D.L.
0.01 LD50
0.001 LD50
1
7
14
21
28
56
Days After Injection

2) Recovery from Moderate and Low Doses

After moderate to very low doses (0.1 to 0.001 LD_{50}), the junctional ultrastructure of diaphragm muscle at 1, 7, 14, 28, and 56 days PI was similar to that of normal or sham injected controls. Figures 36-43). (Figures depicting recovery at 1 day PI from 0.1 -0.001 LD_{50}, are presented in the "Delayed Effects" section, p81-86.) Figures 36-43 (following pp) documented that there were no obvious changes in endplate fine structure at 7,14,28 or 56 days PI at 0.1 to 0.001 LD_{50}. None of the fibers in diaphragm, soleus, or EDL exhibited supercontraction or any other evidence for myofiber damage at 1-56 days (Fig. 31). Similarly, at low and very low doses (0.01 and 0.001 LD_{50}, 10-30% and 0-20% enzyme inhibition, respectively), no evidence for pre- or post-synaptic alterations were observed at 1-56 days post injection.

Since no acute damage was observed at these lower doses, we are now searching for any subtle evidence of delayed toxicity.

b. Recovery from Subacute Exposure (high and low doses)

No long term effects on neuromuscular ultrastructure or physiology were noted at 3, 7, 14 or 28 days after termination of 14 days subacute exposure at low or high doses (30% and 80% inhibition, Figs. 44-47). Myofibers appeared normal, as did all pre- and post-junctional organelles. Thus, long-term effects on muscle ultrastructure and physiology appear minimal following prolonged (subacute) exposure to relatively high doses of physostigmine.

c. Physiology:

For the 10 animals tested at 3, 7, and 14 days after pump removal (6 high dose, 4 low dose), EDL twitch tensions and tension ratios for 20 hz contraction were normal. Whatever minor physiological effects were noticeable after 14 days of pump implantation (see Fig. 11) had been reversed soon after pump removal.

In conclusion, recovery from both high and low dose subacute exposure appears to be rapid and complete, at least as measured physiologically and ultrastructurally at the neuromuscular junction.

Figure 36. Recovery of diaphragm (Fig. 36a), soleus (Fig. 36b), and EDL (Fig. 36c) neuromuscular junctions 7 days after a single acute moderate-dose physostigmine (0.1 LD_{50}) injection. Endplate and myofiber morphology appear normal. No structural alterations were detected in any of the muscles treated at this dose.


DIAPHRAGM
AM-R7
36
SOLEUS
EDL
1
C

Figure 37. Recovery of diaphragm (Fig. 37a), soleus (Fig. 37b), and EDL (Fig. 37c) neuromuscular junctions 14 days after moderate-dose physostigmine (0.1 LD_{50}) exposure. Myofibers, mitochondria, and nerve terminals appear normal.

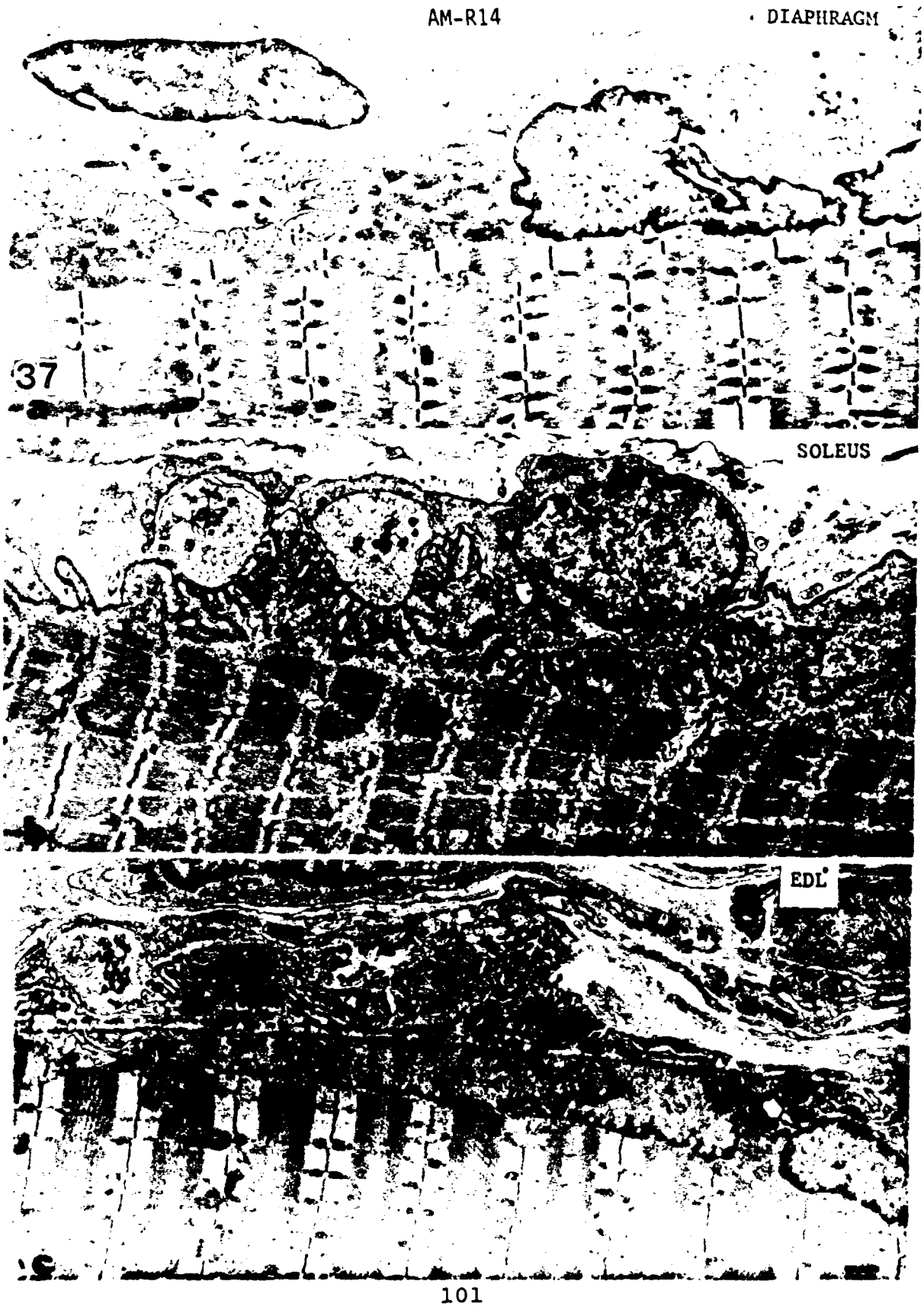

DIAPHRAGM
37
SOLEUS
EDL

Figure 38. Recovery of diaphragm neuromuscular junction 28 days after moderate-dose physostigmine (0.1 LD_{50}) exposure. No detectable alterations were found in the myofibrils, mitochondria, or nerve terminal.

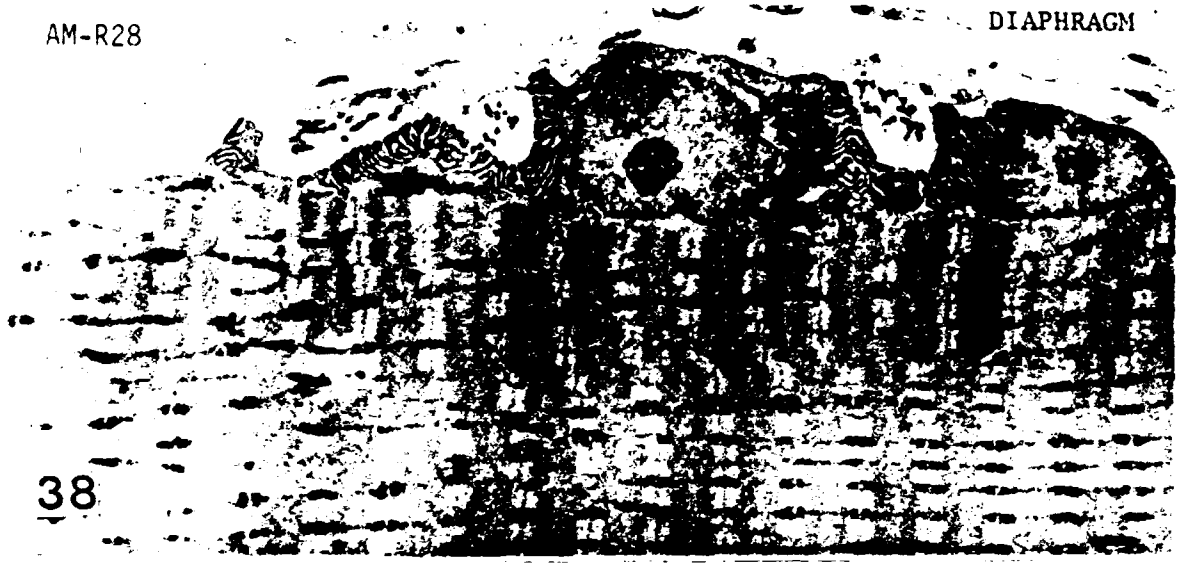

AM-R28
DIAPHRAGM
38

Figure 39. Recovery of diaphragm (Fig. 39a), soleus (Fig. 39b), and EDL (Fig. 39c) neuromuscular junctions 56 days after exposure to a moderate dose of physostigmine (0.1 LD_{50}). Myofibers, mitochondria, and nerve terminals appear normal. The few swollen mitochondria are believed to represent fixation artifacts.

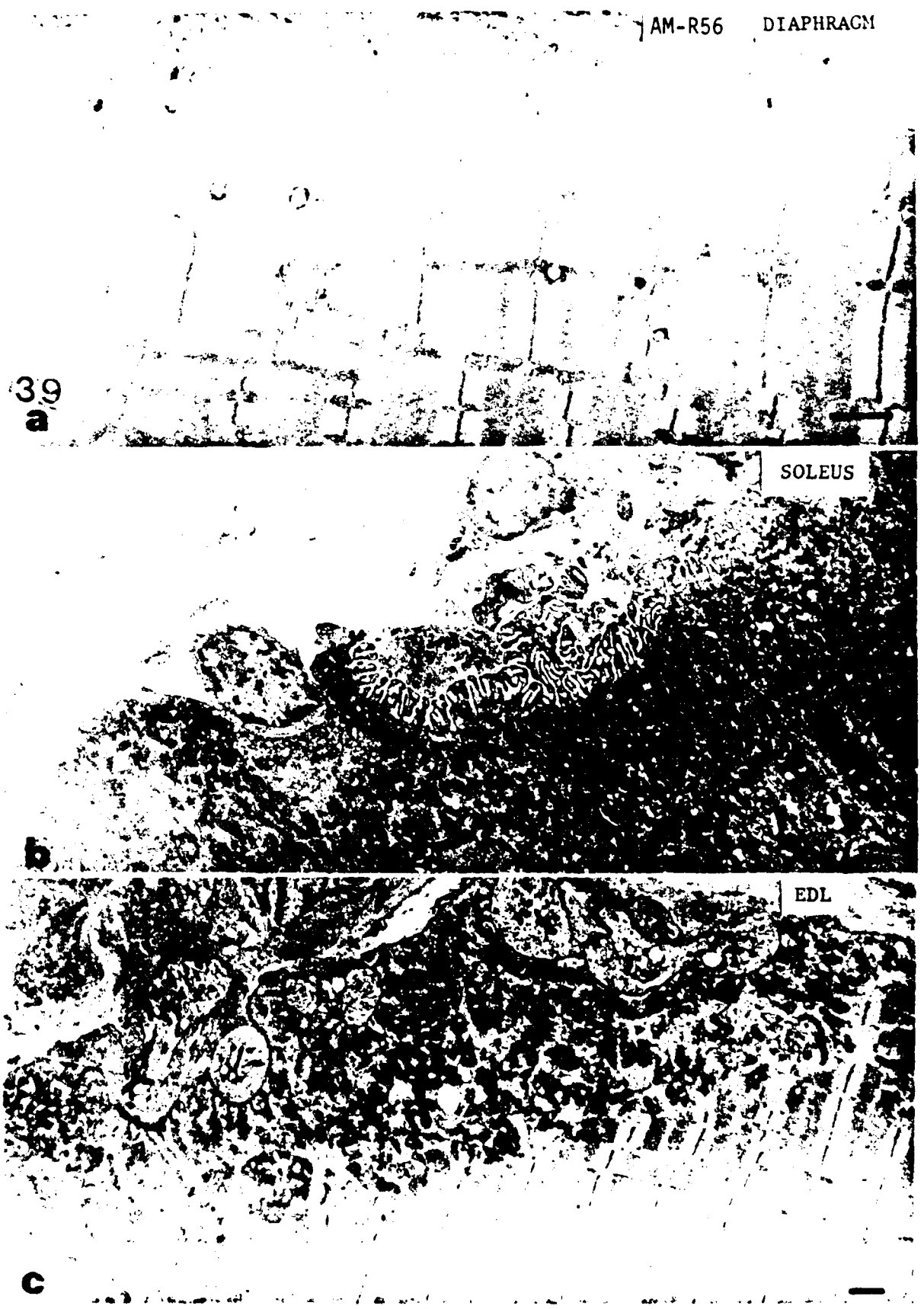

AM-R56 DIAPHRAGM
39
a
SOLEUS
b
EDL
c

Figure 40. Recovery of diaphragm (Fig. 40a), soleus (Fig. 40b), and EDL (Fig. 40c) neuromuscular juncitons 7 days after low-dose physostigmine (0.01 LD_{50}) exposure. Myofibers, mitochondria, and nerve terminals appear normal.

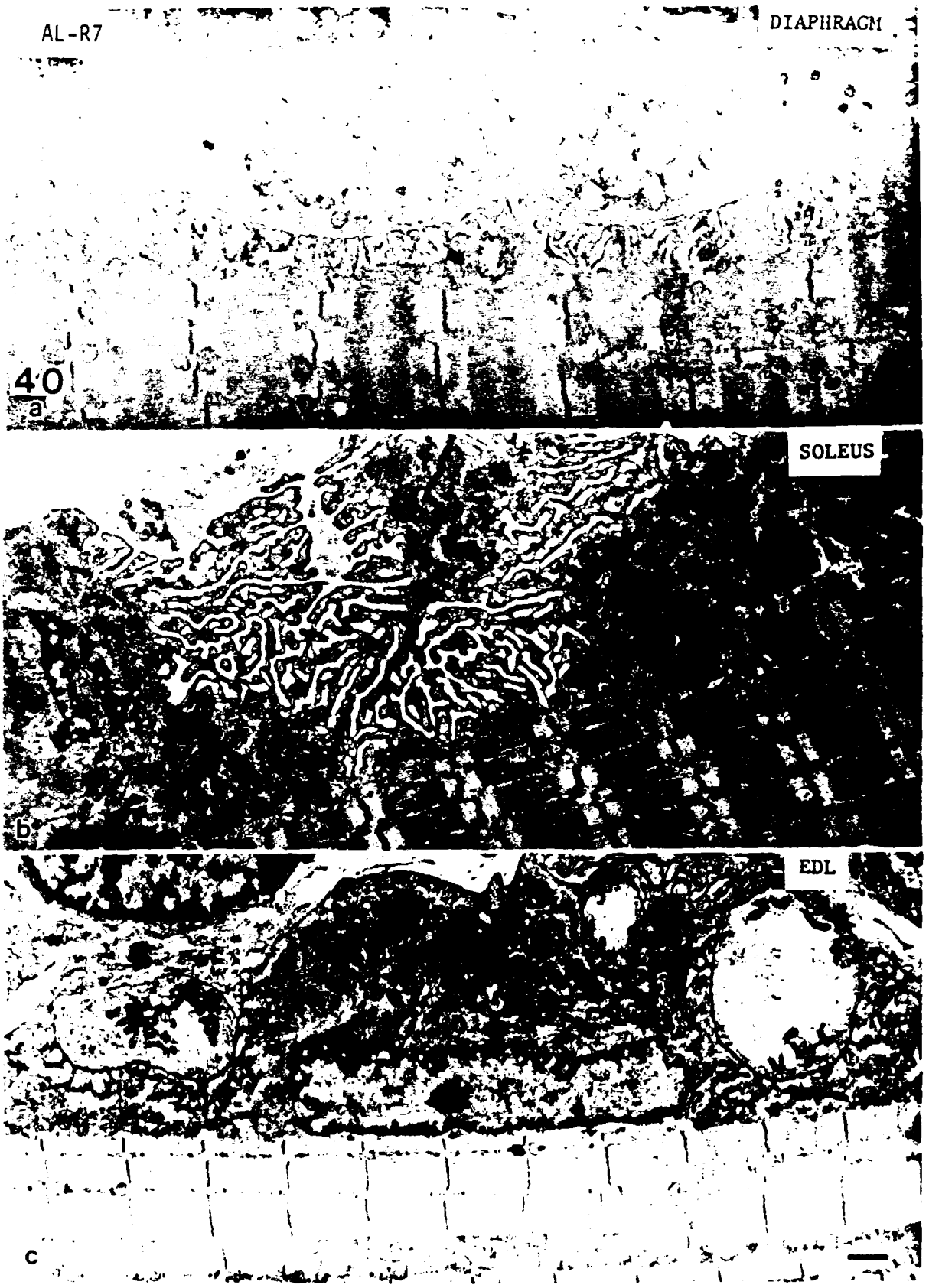

AL-R7
DIAPHRAGM
40
a
SOLEUS
b
EDL
c

Figure 41. Recovery of diaphragm neuromuscular junctions 14 days (Fig. 41a), 28 days (Fig. 41b), and 56 days (Fig. 41c) after low-dose physostigmine (0.01 LD_{50}) exposure. Myofibers, mitochondria, and nerve terminals appear normal.

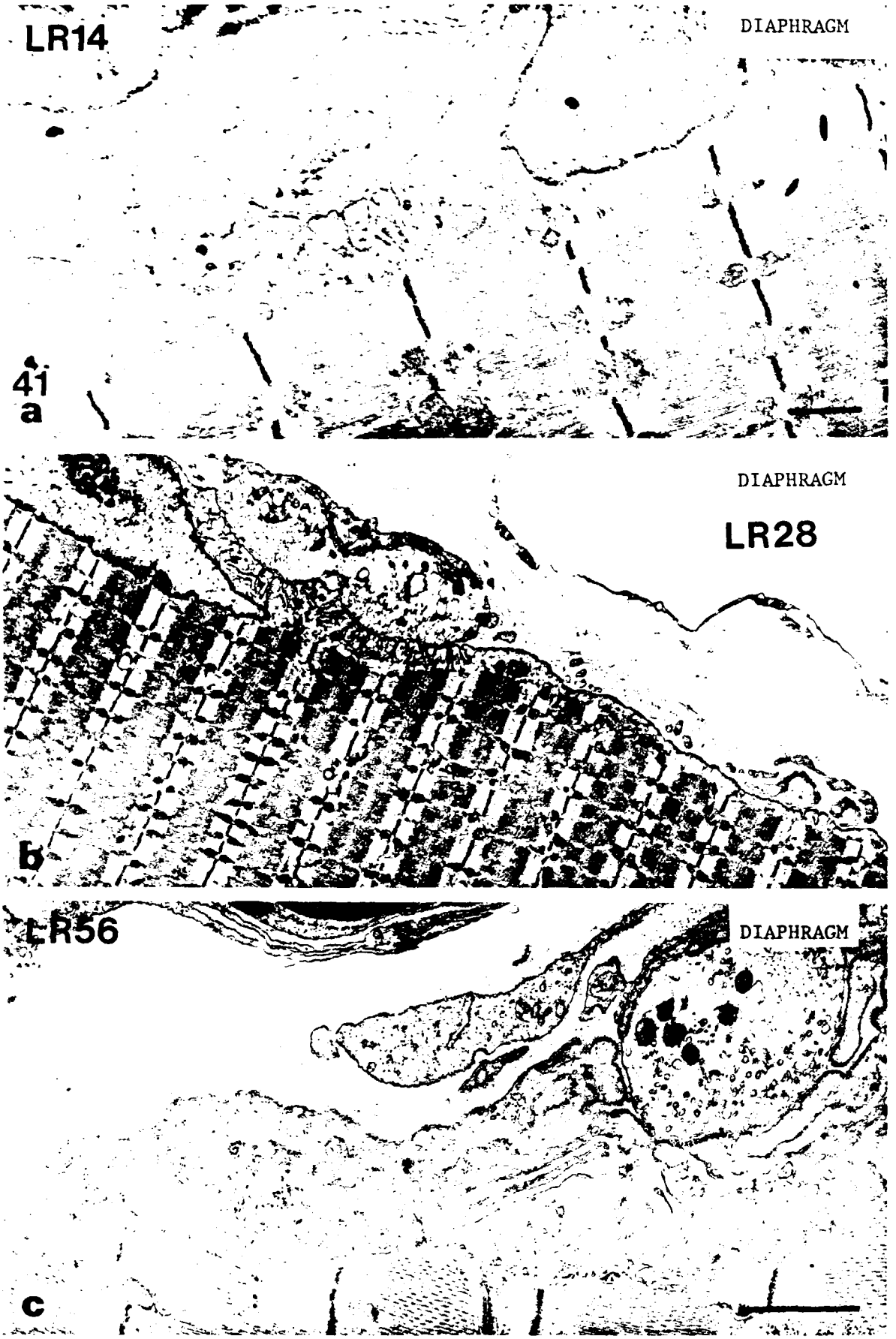

LR14
DIAPHRAGM
41
a
DIAPHRAGM
LR28
b
LR56
DIAPHRAGM
c

Figure 42. Recovery of diaphragm neuromuscular junction 7 days after exposure pf very low-dose of physostigmine (0.001 LD_{50}). Myofibrils, mitochondria, and nerve terminals appear normal.


VLR7
DIAPHRAGM
42

Figure 43. Recovery of diaphragm neuromuscular junctions 14 days (Fig. 43a), 28 days (Fig. 43b), and 56 days (Fig. 43c), after very low-dose physostigmine (0.001 LD_{50}) exposure. Myofibrils, mitochondria, and nerve terminals appear normal.

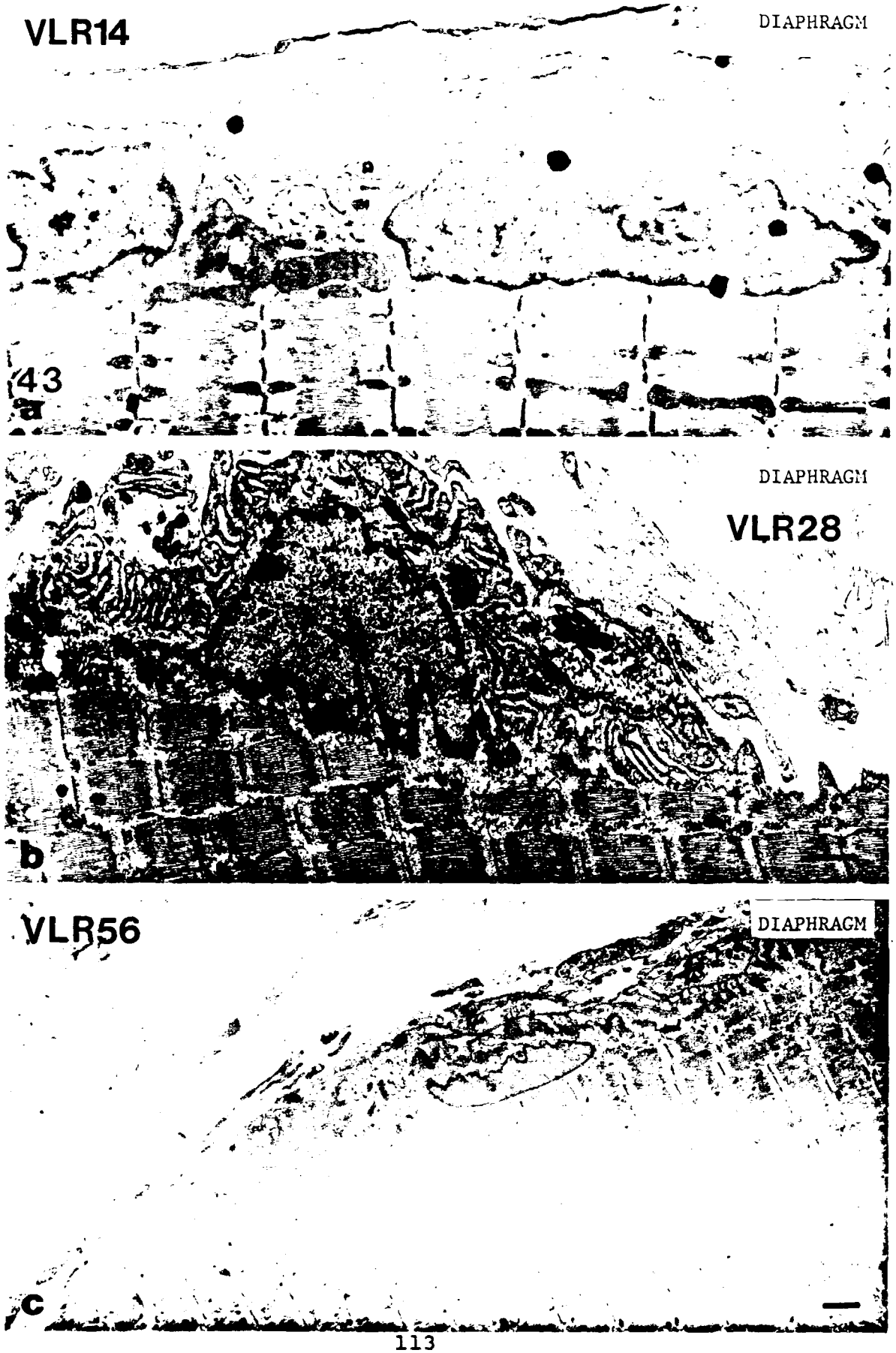

VLR14
DIAPHRAGM
43
a
DIAPHRAGM
VLR28
b
VLR56
DIAPHRAGM
c

Figure 44. Neuromuscular junctions of diaphragm (Fig. 44a), soleus (Fig. 44b), and EDL (Fig. 44c) 14 days after exposure to a low dose of physostigmine (30% serum ChE inhibition) followed by 3 days of recovery (no drug, SL14R3). All myofibers and nerve terminals appeared normal. (Swollen mitochondria in the soleus and EDL are believed to be fixation artifacts. See Lee, et. al., 1986).

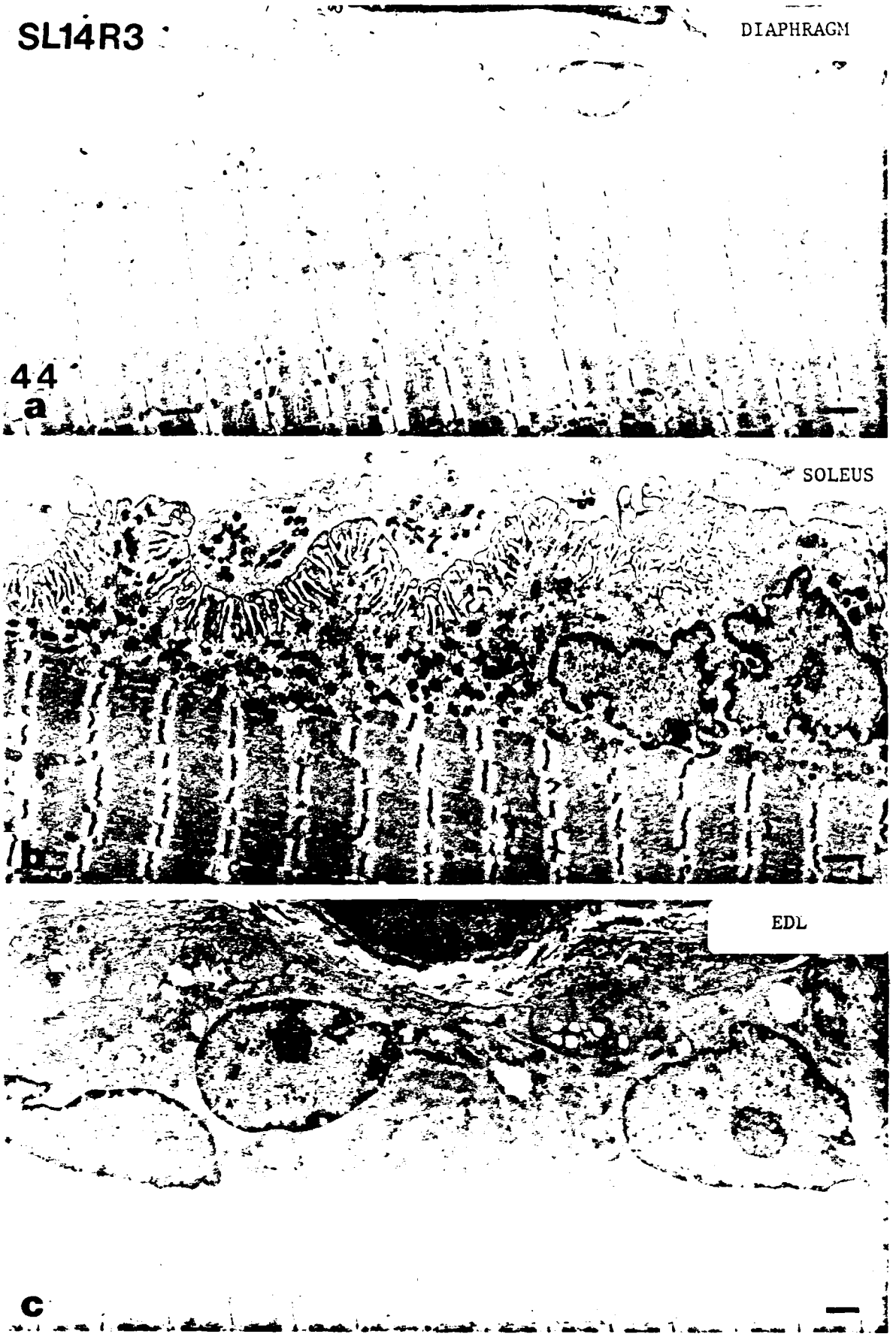

SL14R3
DIAPHRAGM
44
a
SOLEUS
b
EDL
c

Figure 45. Diaphragm neuromuscular junctions after 14 days exposure to a subacute low dose of physostigmine (30% serum ChE inhibition) followed by recovery (no drug) for 7 days (Fig. 45a, SL14R7), 14 days (Fig. 45b, SL14 R14), and 28 days (Fig. 45c, SL14R28). All myofibers, mitochondria, and nerve terminals appeared normal.

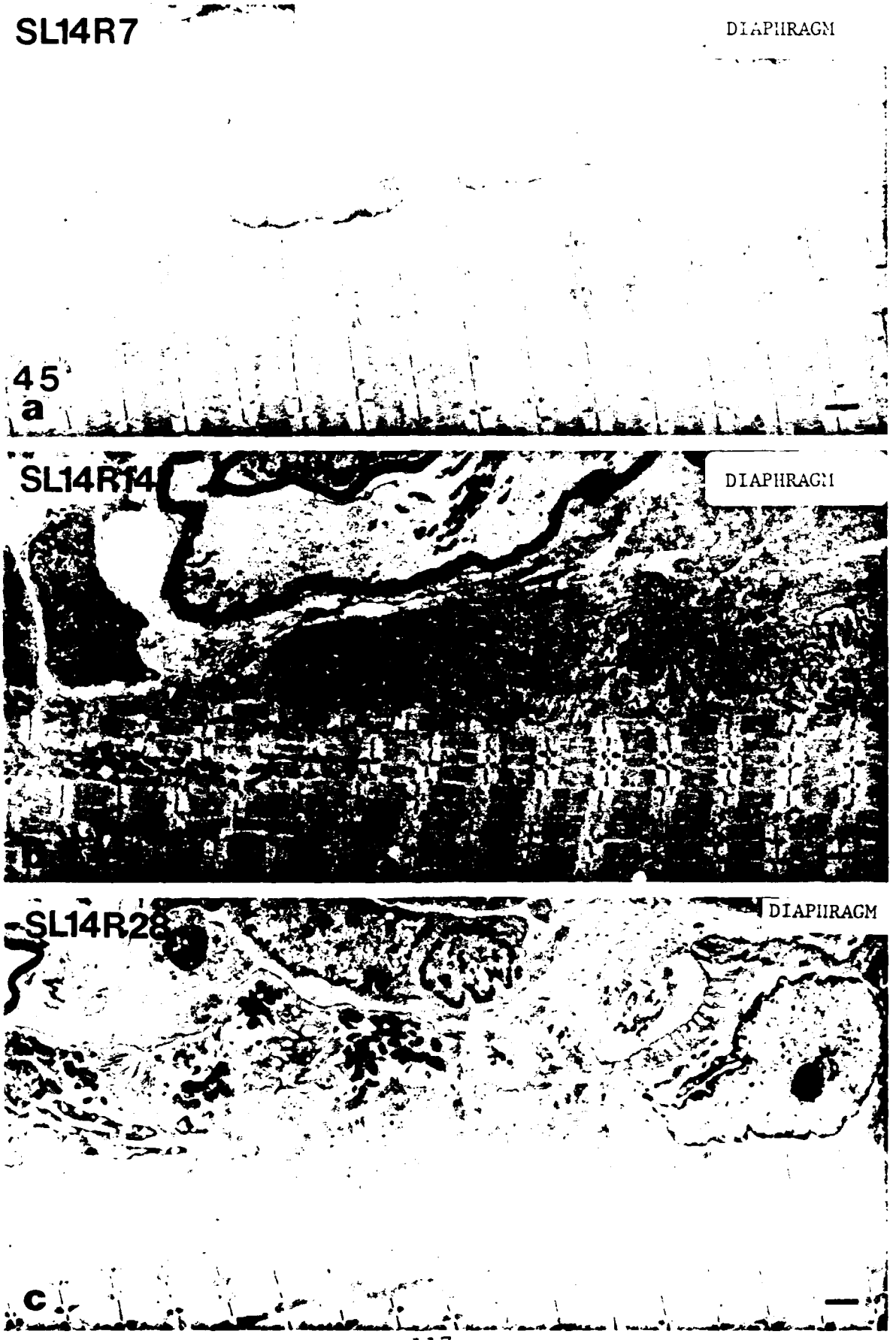

SL14R7
DIAPHRAGM
45
a
SL14R14
DIAPHRAGM
SL14R28
DIAPHRAGM
c

Figure 46. Recovery of diaphragm neuromuscular junctions for 3 days (Fig. 46a, SH14R3), 7 days (Fig. 46b, SH14R7), and 14 days (Fig. 46c, SH14R14) following termination of 14 days continuous subacute high-dose exposure to physostigmine (80% serum ChE inhibiton). All myofibers, mitochondria, and nerve terminals appeared normal.

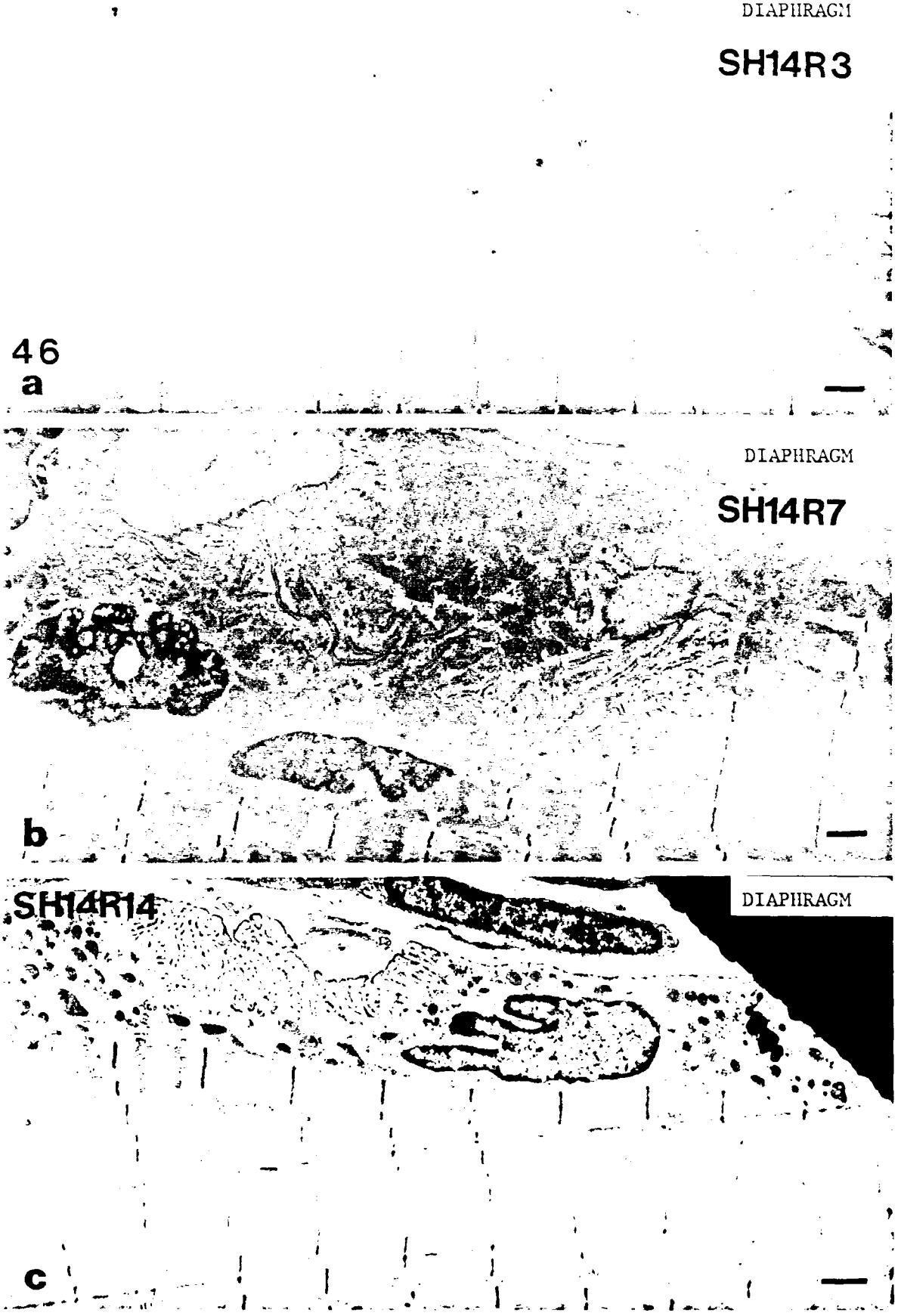
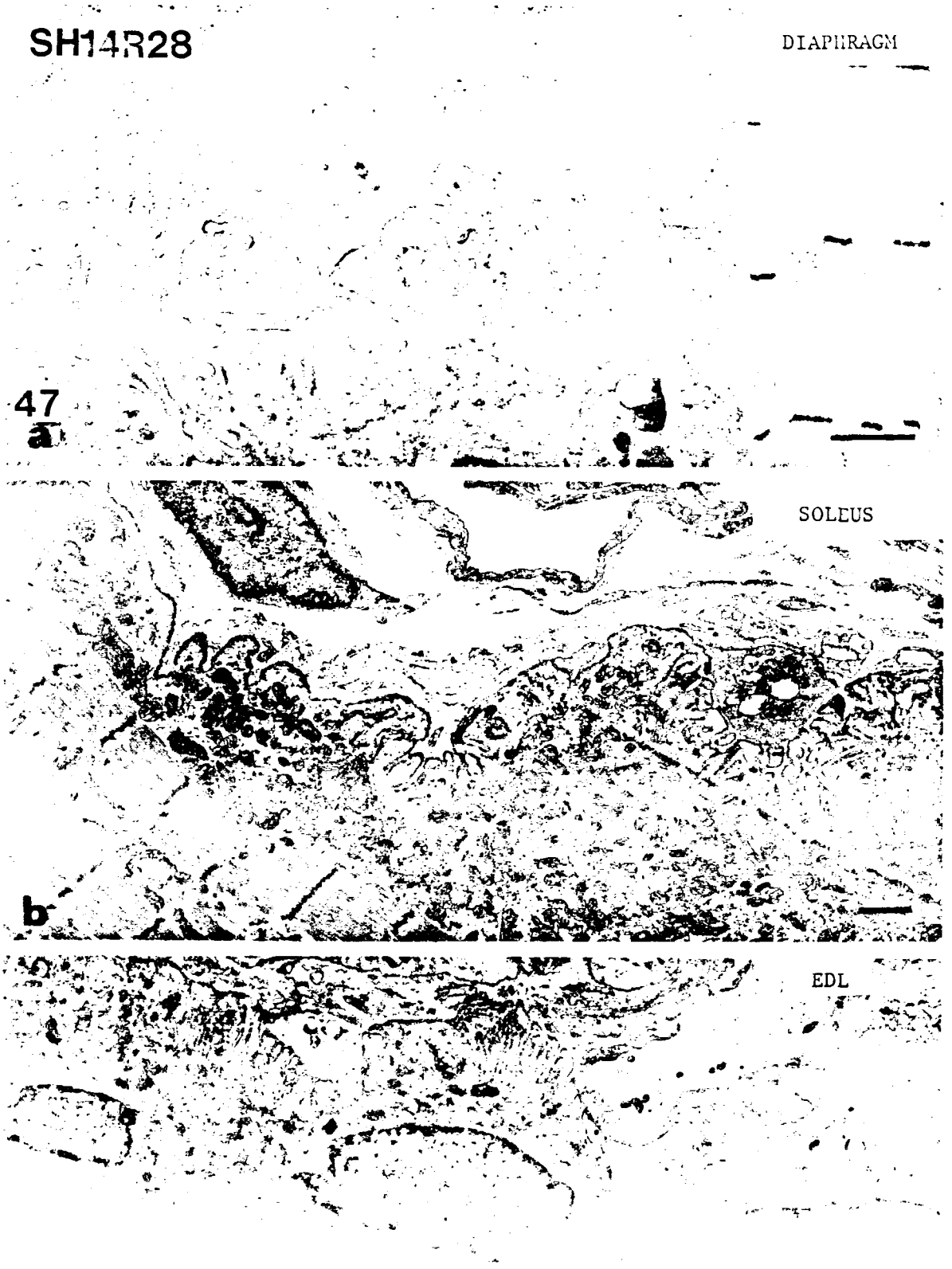

DIAPHRAGM
SH14R3
46
a
DIAPHRAGM
SH14R7
b
SH14R14
DIAPHRAGM
c

Figure 47. Neuromuscular junctions of diaphragm (Fig. 47a), soleus (Fig. 47b), and EDL myofiber (Fig. 47c) after 14 days exposure to physostigmine (80% serum ChE inhibition) followed by 28 days of recovery (no drug, SH14R28). All myofibers, mitochondria, and nerve terminals apppeared normal.


SH14R28
DIAPHRAGM
47
a
SOLEUS
b
EDL
c

G. INTERPRETATIONS

1. OVERVIEW

In this second Annual Report, we have provided more detailed descriptions of the acute, delayed, and long-term effects on mammalian nerve, muscle, and neuromuscular junction ultrastructure and physiology following very low to high dose exposure to the anticholinesterase agent, physostigmine. We have described dose response curves for enzyme inhibition, ultrastructural pathology, and altered physiology. We have also presented data from studies of drug habituation and reversibility and data concerning the effects of subacute exposure. Finally, we have described direct evidence for the effect of muscle use on the threshold of drug-induced pathology.

Enzyme inhibition profiles were maintained on each of the approximately 250 rats prepared for ultrastructural analysis (over 2,900 enzyme assays). Thus, short-term and long-term changes in blood ChE activity/inhibition levels coud be correlated with changes in nerve and muscle ultrastructure and physiology in the same animal. Dose response curves for blood ChE enzyme inhibitions revealed a log-linear relationship over the range 0.01 to 1.1 LD_{50} (with the data at 0.001 LD_{50} considered to be less reliable). Enzyme inhibitions were measured at 33% ± 15% for 0.01 LD_{50}, 67% ± 7% for 0.1 LD_{50}, 75% ± 5% for 0.3 LD_{50}, 89% ± 3% for 0.8 LD_{50}, and 95% at 1.0 LD_{50}. Levels of enzyme inhibition up to 67% were not associated with major changes in myofiber ultrastructure in EDL, soleus, or diaphragm myofibers. Higher doses produced increasingly profound changes in endplate structure and physiology. We note, however, that since the serum ChE sites are not identical with endplate AChE, it is unlikely that the two enzymes would saturate at the same rate, especially at very low doses. Thus, it is not surprising that blood ChE enzyme inhibition levels are not correlated with detectable changes in endplate ultrastructure until high levels of blood ChE inhibition are reached (70%).

Our data indicate that:

a) physostigmine-induced supercontraction, as well as other changes in myofiber ultrastructure, are dose dependent, with severe damage occurring above a threshold of 74% enzyme inhibition, and

b) delayed reactions to anticholinesterase exposure are relatively infrequent but include partial junctional fold destruction and possible endplate remodeling, partial denervation and reinnervation phenomena, beginning 1 to 2 weeks after a high dose exposure to physostigmine.

In addition, we show:

c) relatively minor alteration of nerve-muscle ultrastructure or physiology following subacute (prolonged) exposure at relatively high dose exposure, with

d) no detectable alteration following low dose subacute exposure, and

e) that muscle use may be an important factor in drug toxicity, habituation, and reversibility.

We have confirmed the occurrence of extremely rapid

mechanisms for plasticity, repair, and recovery of function of nerve terminals, muscle plasma membranes, and muscle cytoplasm. Thus, several of the most obvious cytotoxic effects of physostigmine were shown to be at least partially reversible. However, the occurrence of delayed destructive changes suggests the need for cautious evaluation of these secondary responses, especially as they apply to non-therapeutic uses.

2. TASK I

a. OCCURRENCE OF SUPERCONTRACTION AFTER ACUTE HIGH DOSE EXPOSURE

Thirty minutes after single high dose injections of 0.8 LD_{50}, all neuromuscular junctions of the constantly-used diaphragm and soleus myofibers exhibited supercontraction of sarcomeres in the subjunctional sarcoplasm. Z bands were missing, destroyed, or grossly disrupted. Free thick and thin filaments were present in disorganized masses, and a mixed population of "frothy" and grossly distended mitochondria plus distended sarcoplasmic reticulum cisternae were observed disrupting the subjunctional sarcoplasm. However, EDL muscles from the same rats were much less affected at ½ hour. Although many EDL fibers exhibited "blistered" or "frothy" mitochondria in the immediate subsynaptic regions, most fibers exhibited no detectable changes in sarcomere ultrastructure. Thus, not only have we shown conclusive evidence for supercontraction, but we have also shown that the thresholds for supercontraction occur at 80% for the constantly used diaphragm myofibers, 85% for the postural soleus muscles, and about 90% for the less frequently activated EDL myofibers. (Data suggesting that these apparent fibro-type differences in susceptibility to damage arise instead from differences in muscle use patterns/indirect muscle stimulations are discussed in the next section.)

b. SUPERCONTRACTION IS NOT FIBER TYPE SPECIFIC

Earlier comparisons of soleus (slow twitch) and EDL (fast twitch) have suggested that the slow twitch fibers had more extensive damage to a greater percentage of the fibers than in fast twitch muscle (13,20) and have concluded that the effects of anti-ChE agents are "fiber-type specific," based either on inherent differences in the myofibers or on their tonic (continuous) vs. phasic (intermittent) activation patterns (41). However, our detailed examinations have revealed that in the diaphragm (a mixed muscle containing both fast twitch and slow twitch fibers), all myofiber types demonstrated severe supercontraction at 0.8 LD_{50}. In the soleus (a constantly used postural muscle) even though all myofibers were supercontracted, damage to the subjunctional mitochondria was noticeably less than in the diaphragm. Z bands, for example, were recognizable even in the areas of maximum supercontraction. In contrast, in the infrequently used EDL, damage was not as extensive at 1/2 hour, but most myofibers showed evidence for extensive prior supercontraction by 24 hours PI. Interestingly, many fibers in the diaphragm exhibited severe damage at 0.3 LD_{50}, but similar damage was not observed at that "threshold" level in soleus or EDL.

Since the diaphragm is a mixed muscle and since fast twitch, intermediate, and slow twitch myofibers in that muscle were all equally severely affected, it is clear that the damaging effects are not fiber-type specific. Rather, we observed that the rats were incapacitated during the initial 30 minutes after physostigmine injection and that the EDL muscles were essentially unused for at least ½ hour during the initial period of exposure. Thus, it is not surprising that the EDL fibers showed

substantially fewer ultrastructural defects than diaphragm myofibers (which it should be remembered were used constantly for breathing) or soleus myofibers (which were used to support the animal when standing or walking). Likewise, since these same rats had recovered their respiratory capabilities and exhibited partially repaired diaphragm myofibers, the increased damage to EDL fibers at 24 hours PI appears to be related to the resumption of voluntary myofiber activity during the initial 1-6 hour period of low esterase activity, and not to inherent differences in fast vs. slow twitch muscle. Thus, intrinsic muscle use patterns appear to be related to the threshold and the amount of muscle damage.

The observations and interpretations cited in the preceding paragraph are supported by preliminary experiments in which EDL and soleus muscles are activated at 20, 40, and 80 hz (6 sec duration every 10 sec) in animals treated at 0.1 and 0.3 LD_{50}. Damage to EDL and Sol (including swelling of subjunctional mitochondria, ER, SR, Golgi cisternae and nuclear membranes) was noted at 40 and 80 hz in rats exposed to 0.3 LD_{50}. This is in direct contrast to EDL myofibers in unstimulated animals in which the fibers are little affected, even at doses as high as 0.8 LD_{50}. These preliminary data are consistent with the proposed synergistic effect of anticholinesterase agents and neuromuscular activity.

c. WHICH MUSCLE IS THE BEST MODEL FOR ANALYZING CHOLINESTERASE TOXICITY?

Since the constantly used diaphragm is most sensitive to the pathological alterations caused by physostigmine (see above) and since failure of diaphragm myofibers results in death by suffocation, we suggest that the diaphragm is the best model for analyzing the in vivo effects of anticholinesterase drugs in muscle ultrastructure and physiology. However, due to the ease with which the EDL and Soleus muscles can be activated by indirect (nerve) stimulation, we continue to utilize these muscles for studies of muscle use on altered physiology and on the alteration of threshold of drug toxicity.

d. ACUTE EFFECTS OF PHYSOSTIGMINE ON EDL PHYSIOLOGY

Measurable potentiation of EDL twitch tension began 7 to 30 minutes after subcutaneous injection of physostigmine with time of onset related to time required for the ChE inhibition level to reach 75-80%. Duration of the response ranged from 1 to 46 minutes, the twitch tension returning to normal before the ChE inhibition level dropped below 80%. Maximum twitch potentiation attained apparently was influenced by several factors including peak ChE inhibiton level, rise time to inhibition greater than 80%, duration of inhibition above 80%, resting tension placed on the EDL for recording, and stress response of individual animals.

3. TASK II: EFFECTS OF SUBACUTE EXPOSURE

Analyses of blood ChE enzyme inhibitions were monitored following subacute exposure to physostigmine at very low to high doses (corresponding to 0.003LD_{50}/hr and 0.3 LD_{50}/hr), blood ChE inhibition of about 50% and 70-80% were maintained for 3, 7, and 14 days. This small difference in ChE inhibition resulted from a 100-fold difference in amount of physostigmine delivered. Since 0.3 LD_{50} resulted in 70-80% ChE inhibiton ½hr PI, it was surprising that this same amount was required to be delivered each hour to maintain the same inhibition levels during subacute exposure. This high dose of physostigmine to obtain 70-80% inhibition of ChE may reflect some form of **HABITUATION** since for a single injected dose ChE enzyme inhibition is dependent on the logarithm of drug concentration. Hence, a 1.5 fold increase in ChE inhibition would have been expected to require 10 X more physostigmine but not the observed 100 X greater dose. The blood ChE data from rats with Alzet pumps can be roughly reconciled with the blood ChE data from rats having a single injection if an assumption is made that the half life of physostigmine in the rat changes substantially in a dose dependent manner. Exposing a rat to 0.003 LD_{50}/hr would yield blood ChE levels predicted by single dose injections if the half life of physostigmine is about 6 hrs. But exposure of a rat to 0.3 LD_{50}/hr would yield blood ChE levels predicted by a single dose if the half life of physostigmine in the rat is about 1 hour. (Published estimates suggest a half life of less than 2 hrs, in humans, ref. 1.)

Interestingly, in rats implanted with "partially pre-primed" pumps (which yield a slower rise in physostigmine concentration than in "pre-primed" pumps), there were no supercontracted fibers in diaphragm, soleus, or EDL muscles at any exposure interval up to 14 days of continuous infusion of a "high dose". Some form of habituation is implied here also because a single subcutaneous injection (i.e., a "bolus") of 0.3 LD_{50} produced supercontraction in 60% of diaphragm myofibers, yet 24 doses of 0.3 LD_{50} delivered at a rate of one per hour (i.e., 7 LD_{50}/day) yielded no supercontraction in any fibers. Since serum ChE inhibitions rise somewhat more slowly following implantation of Alzet pumps than following direct subcutaneous injection, the appearance of supercontraction in injected animals but not in Alzet pump implanted animals appears to be due to the higher initial concentration obtained by injection and/or the rate of rise of physostigmine concentration. In either case, it is clear that blood/ChE inhibition levels do not necessarily reflect endplate inhibition levels and that some form of "habituation" occurs during gradual endplate ChE inhibition.

In support of this notion is the observation reported in our First Annual Report that the use of "pre-primed" pumps resulted in 100% lethality at a much lower drug delivery rate (i.e., at 0.1-0.3 LD_{50}/hr). Here again, differences in the rate of initial delivery or enzyme binding combined with normal respiratory activity produce profound differences in lethality/drug effect.

These studies warrent further examination of habituation kinetics and the mechanisms of habituation.

Physiology

For subacute exposures, EDL twitch tensions and high frequency contraction were not significantly different from normal. Since high dose pumps produced sustained ChE inhibitions of 80%, some damage to EDL neuromuscular junctions would have been expected. However, since the inhibiton levels did not reach 90% (threshold for EDL supercontraction), apparently the limited physiological damage was not severe enough to substantially affect whole muscle function. The only observed effect on EDL physiology was a slight tendency towards greater fatigability during high dose treatment. This is in contrast to results of Adler et. al. (1984), who reported substantially increased EDL twitch tension with single stimuli, and depression with 20 hz stimulation during subacute administration of pyridostigmine. Apparently, for damage sufficiently severe to affect whole muscle function in the EDL, the threshold is higher for physostigmine than for pyridostigmine.

Although the threshold for onset of twitch potentiation in the acute studies was at a ChE inhibiton of about 80%, sustaining that level of inhibition does not necessarily sustain twitch potentiation. This was shown in the acute experiments where twitch tensions returned to normal while blood ChE inhibition levels were still well above 80%. Also, in the subacute experiments in which EDL physiology was tested while the pumps were still in place, normal twitch tensions were recorded when the ChE inhibition was greater than 80%. Later, with no significant change in inhibition level, substantial twitch potentiation occurred. These examples emphasize that threshold for physostigmine-induced damage is dependent on the interaction of several factors.

4. TASK III.

a. DOSE RESPONSE AFFECTS OF ACUTE EXPOSURE

In the First Annual Report, we showed that supercontraction does not occur at or below 0.1 LD_{50} but that most fibers in diaphragm and soleus had supercontracted myofibers in the subjunctional sarcoplasm at 0.8 LD_{50}. In this Second Annual Report, we present a refined dose response curve showing that 60% of diaphragm myofibers are supercontracted at 0.3 LD_{50} whereas unstimulated soleus and EDL are not seriously affected. Thus, we define 0.3 LD_{50} as the "threshold" dose for gross cytopathology. Moreover, we showed that increased endplate activity induced endplate cytopathology in soleus and EDL, with maximum cytopatholgy in soleus occuring following prolonged stimulation at 20 hz. In contrast, in the EDL, cytopathological alterations increased with increasing stimulation frequency. (Maximum rates tested were 80 hz.) Thus, "dose-response" curves are somewhat misleading unless levels of musclular activity are specified or controlled. Likewise, any statement concerning cholinesterase toxicity/lethality must also recognize the synergism of muscle use vs. drug exposure.

b. ACUTE-DELAYED EFFECTS

For doses of 0.8-1.1 LD_{50}, the destructive effects observed in all myofibers of diaphragm and soleus fibers were partially reversed 24 hours PI, and blood ChE levels had returned to near normal. In contrast, at 24 hours PI, EDL fibers exhibited increased myofiber damage and increased "frothy" and partially distended mitochondria. (For a discussion on fiber-type specificity, see First Annual Report.) Seven days after injection of 0.8-1.1 LD_{50}, junctional folds in some diaphragm and soleus myofibers were devoid of attached nerve terminal branches. By 14 days PI, small diameter nerve processes were observed, some <u>not</u> embedded within a primary synaptic cleft. In some endplates, junctional folds were missing and were replaced by vesicular debris similar to that seen after exposure to other anticholinesterase agents and in myasthenia gravis. These endplate alterations suggest that in the most severely affected fibers, a process of (partial) denervation and reinnervation via small collateral "sprouts" had occurred. Dose response curves for these morphological alterations were presented in Figures 26, 27 and 34. Those data graphically revealed that extreme morphological damage occurs only at very high doses of physostigmine (0.8-1.1 LD_{50}) or at moderate doses (0.3 LD_{50}) only after sustained neuromuscular activity (i.e., normal respiration or indirect stimulation). Since twitch potentiation responses were obtained in EDL muscles that were treated at doses below threshold for producing supercontraction only when they were stimulated relatively frequently, these data are consistent with suggestions that muscle use exacerbates the effects of anticholinesterase exposure.

5. TASK IV: REVERSIBILITY

a) acute-recovery

We showed that enzyme inhibition is rapidly completely reversible after a single acute dose of physostigmine (Fig. 1). Following termination of exposure, blood ChE rapidly returned to normal, or in many cases, rebounded to a higher than normal level. The ramifications of this heightened blood ChE level are not known, nor is it known if endplate ChE follows a similar time course. Nevertheless, it is possible that there are long-term compensatory changes in endplate ChE enzyme profiles that are not detected by these methods.

Ultrastructurally, there is rapid recovery from the major damage occurring during the first 24 hours. By 7 days, there is little remaining evidence for supercontraction; mitochondria appear normal; and the sarcoplasmic reticulum appears normal. However, in the First Annual Report, we also showed that long-term changes in endplate morphology occurred in some myofibers ("simplification" of junctional folds, formation of vesicular membrane debris in place of some junctional folds, disappearance of nerve terminal branches, formation of collateral sprouts, and the apparent formation of new regions of nerve-muscle apposition in and near the original endplates). We concluded then that physostigmine may exert substantial influences on biological control mechanisms regulating endplate morphology.

The physiological effects being measured (EDL twitch potentiation and sustained high frequency contraction) returned to normal within one hour after acute physostigmine administration. In all cases, this return to normal physiology occurred when blood ChE inhibition levels were still 80% or higher. The relative extent of damage done to EDL during this period was readily reversible at least in terms of whole muscle physiology. For the subacute/recovery exposures, at 3 days recovery and thereafter, EDL twitch tension and high frequency contraction properties were normal. This would be expected because a) ChE inhibition levels had returned to 0% (i.e., normal) and b) because these physiological properties were minimally altered in the period before recovery.

b) subacute recovery.

There were no detectable changes in endplate or muscle ultrastructure within one day following termination of subacute exposure, nor were any changes noted up to 28 days later. Thus, recovery from the slight alterations noted following 14 days of high dose subacute recovery seems rapid and complete in so far as it can be measured at the neuromuscular junction.

H. CONCLUSIONS AND RECOMMENDATIONS

1. We have demonstrated destructive supercontraction occurs at near lethal high doses of physostigmine, and that the threshold for producing supercontraction in muscles used for respiration is about 0.3 LD_{50}.

2. We have demonstrated that in the rat there is extremely rapid recovery from severe ultrastructural damage to myofibers produced by near lethal doses of physostigmine.

3. We have demonstrated that there are essentially no ultrastructural alterations produced in myofibers by moderate to very low acute doses of physostigmine. Previous reports suggesting mitochondrial alterations at low to very low doses of other anticholinesterase drugs are tentatively attributed to fixation artifacts rather than to drug effect.

4. We have shown that subacute administration of moderate to high doses of physostigmine does not produce significant changes in maximum muscle twitch tension but that resistance to fatique may be lowered at moderate to high doses.

5. In preliminary experiments (not required by this contract), we have shown that under normal physiological stimulation conditions (low stimulation frequencies and/or short durations of stimulation), there were no ultrastructural alterations of soleus or EDL muscles. However, with sustained moderate to relatively high stimulation, gross muscle pathology was produced at what are normally "subthreshold" doses.

6. We have also shown that subacute ("chronic") exposure to physostigmine at low to moderate doses produces few detectable alterations in endplate fine structure.

Physostigmine has been suggested as a possible therapeutic agent for the protection of military personnel against exposure to the toxic nerve agents. Our data are compatible with this suggestion. However, before any chemotherapeutic agent is selected, our data also suggest the need for direct comparison of the effects of this agent (physostigmine) with other agents (i. e., pyridostigmine) prior to the use of either for chemoprophylaxis.

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GLOSSARY

A band That portion of the sarcomere corresponding to the region of thick filaments.
AChE/acetylcholinesterase The enzyme which hydrolyzes acetylcholine.
ACh receptor Chemically sensitive ion channels localized on the crests of the junctional folds. They respond to acetylcholine by opening and allowing ions to cross the muscle plasma membrane at the NMJ.
Anticholinesterase Chemicals that bind to and block the action of cholinesterases (including AChE and butyrylChE)
I band That portion of a sarcomere containing only thin filaments.
Junctional folds The specialized infoldings of the sarcolemma which contain high densities of ACh receptors.
Motor endplate Equivalent to neuromuscular junction (see).
Myofiber A muscle cell. (Not equivalent to a myofibril, see below.)
Myofibril A contractile strand of sarcomeres. There are several hundred myofibrils in each myofiber.
Myofilaments Each sarcomere (see below) consists of hundreds of thick (myosin) filaments and thin (actin) filaments. Not to be confused with myofibers (cells) or myofibrils (chains of sarcomeres).
Neuromuscular junction The region of apposition of the motor nerve and muscle plasmamembranes, the site where the muscle receives the chemical message from the nerve.
Physostigmine A reversible tertiary amine carbamate antiChE.
Pyridostigmine A reversible quartenary amine carbamate antiChE.
Sarcolemma The muscle cell plasmamembrane (plus associated external coats of glycoproteins).
Sarcomere The fundamental repeating unit of the myofibril; one sarcomere is the portion of a myofibril from one Z band to the next Z band.
Sarcoplasmic reticulum A network of membranes surrounding each myofibril, one component surrounds the A band while a second surrounds the I and Z bands. The two elements are coupled at the "triad" via the T-system tubules (see T-system and "triad").
Supercontraction A pathological state where sarcomeres in (a portion of) the myofibril contract to the point that thick filaments are thrust against or into the Z bands, resulting in crumpling and/or disassembly of thick filaments. Sarcomere dissolution often accompanies supercontraction.
T system A branching system of tubules that are continuous with and conduct the muscle action potential from the sarcolemma.
Triad The three-part junction of one T-tubule and the two components of the sarcoplasmic reticulum.
Twitch tension The maximum force exerted by an entire twitch muscle following a supramaximal stimulation of the innervating nerve bundle.
Tension ratio The ratio of the tension generated at the beginning of a high frequency stimulation divided by the tension at the end of the stimulus train.
Z band The very narrow electron dense bands traversing the myofibril separating into sarcomeres.

TABLE A. % ACh Activity-Control (no drug)

Animal #	Day 1	7	8	9	14	PUMP OUT //	17	28
S-263	75			107	99	//		90
S-267	94	87			101	//	93	
S-275	121		108		95	//		
AVG.	97	87	108	107	98	//	93	90
±S.D.	23	-	-	-	4	//	-	-
N=	3	1	1	1	3	//	1	1

AVG. For Total Data

(PUMP IN) 99
±4
N=9

(PUMP OUT) 92
N=2

SUM TOTAL AVG.
97
±12
N=11

TABLE B. % ACh Activity-Low Dose
(0.25 mg/ml @ 25 µl/hr)

Responding Animals

Animal #	1	3	7	8	14	PUMP OUT	3
S-268	39			35	35	//	
S-269	69			52	46	//	
S-270	50			40	42	//	84
S-272	44			48	35	//	
S-273	56			63	72	//	
S-274	41			39		//	
S-287	75	28				//	
S-289	68				109	//	
S-290	46		24		9	//	
AVG.	54	28	24	46	50	//	84
±S.D.	13	-	-	10	32	//	
N=	9	1	1	6	7	//	1

Non-Responding Animals

Animal #	1	3	7	8	14	PUMP OUT	3
S-260	131			120	139	//	217
S-261	88			86	75	//	109
S-262	144		96		107	//	156
AVG.	121		96	103	107	//	161
±S.E.	29	-	-	24	32	//	54
N=	3		1	2	3	//	3

TABLE C. % ACh Activity after High Dose (25mg/ml @ 25 µl/hr)

Animal #	Day 1	2	3	4	7	10	13	14	21	PUMP OUT	3	7	14	28
S-234†	20	16	35							//				
S-239	40			20		32		14		//	138			
S-246*	70	57						29		//				91
S-247	21		24							//				
S-248	25		13							//				
S-250*	24		18		15					//				
S-251	29		22		41					//				
S-252	29		27		31					//				
S-253	24		23		29			25		//				
S-254	16		56		22			17		//				
S-255	26		26		32			29		//				
S-256	36		35		26					//			193	
S-257	29					36	21			//			158	
S-258	21					25	17			//			118	
S-264	24				41			17		//		114		
S-266*	20				17					//				
S-276	23				16			19		//	94			
S-277	27				23			15		//	114			
S-278	25				30			22		//	79			
S-291*	24				23			20		//				
S-292	42				53			37	20	//				
AVG.	28	37	28	20	29	31	19	22	20	//	106	114	156	91
± S.D.	14	-	13	-	11	5	-	17	-	//	26	-	38	-
N=	21	2	10	1	14	3	2	11	1	//	4	1	3	1
% inhib.	72	63	22	80	71	69	81	78	80	//	0	0	0	-

* Animal developed capsule around injection site
† This animal @ 30 mg/ml

TABLE D: **Summary of % ACh Activity Data**

DOSE	Day 1	2	3	4	7	8	9	10	13	14	21	PUMP OUT	3	7	14	28
CONTROL	97				87	108	107			98		///			93	
±S.D.	23									4		///				
N	3				1	1	1			3		///			1	
HIGH DOSE	28	37	28	20	29			31	19	22	20	///	106	114	156	91
±S.D.	14		13		11			5		17		///	26		38	
N	21	2	10	1	14			3	2	11	1	///	4	1	3	1
LOW DOSE												///				
Response	54		28		24	46				50		///	84			
±S.D.	13					10				32		///				
N	9		1		1	6				7		///	1			
No resp.	121				96	103				107		///	161			
±S.D.	29									32		///	54			
N	3				1	2				3		///	3			

Over-all Control = 97 ±12 N = 11

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